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Proceedings of the Fifth Annual Chemical Defense Bioscience Review

US Army Medical
Research and Development Command

Appendix 2

Sponsored by the

US Army Medical Research
Institute of Chemical Defense

29 - 31 May 1985

FOREWORD

In conducting the research described in these proceedings, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.

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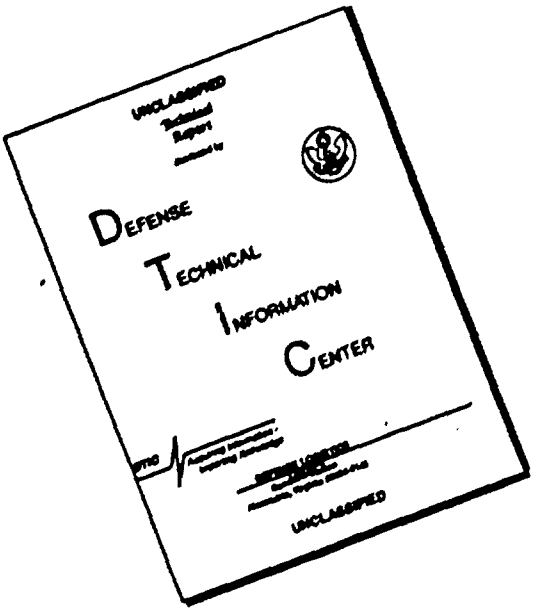

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Proceedings of the Fifth Annual Chemical Defense Bioscience Review

US Army Medical
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Appendix 2

Johns Hopkins University Applied Physics Laboratory
Columbia, Maryland

29 - 31 May 1985

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The Fifth Annual Chemical Defense Bioscience Review was held at Johns Hopkins University Applied Physics Laboratory, Columbia, Maryland on 29-31 May 1985. The objective of this review was to present selected research findings in the area of medical defense against chemical warfare agents. This document constitutes the proceedings of that review. Contents of this volume include:

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7. NEUROTRANSMITTER EFFECTS

7. NEUROTRANSMITTER EFFECTS (continued)

4. Physiology

THE RELEASE OF HISTAMINE FROM HUMAN BASOPHILS APPEARS TO BE REGULATED BY AN ESTERASE AND A MUSCARINIC-LIKE RECEPTOR

Henry Louis Meier, Clark L. Gross, and Bruno Papirmeister
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Aberdeen Proving Ground, Maryland 21010-5425

OVERALL HYPOTHESIS

1. WHEN BASOPHILS ARE ACTIVATED, THEY PRODUCE AN INTRACELLULAR ESTER WHICH REACTS WITH A MUSCARINIC-TYPE RECEPTOR, RESULTING IN HISTAMINE RELEASE.

2. LOW LEVEL PRODUCTION OF THE ESTER DOES NOT CAUSE HISTAMINE RELEASE BECAUSE OF AN ESTERASE WHICH CLEAVES THE ESTER BEFORE IT CAN REACT WITH THE RECEPTOR.

3. DESENSITIZATION OF BASOPHILS COULD RESULT FROM THE ESTERASE CLEAVING THE ESTER BEFORE CALCIUM IS PRESENT TO PERMIT RELEASE.

4. ISOLATION OF BASOPHILS FROM PLASMA RESULTS IN MINIMAL ACTIVATION OF THE BASOPHIL AND THE LOW LEVEL PRODUCTION OF ESTER WHICH WOULD NORMALLY BE CLEAVED BY THE ESTERASE.

5. EXPOSURE OF CELLS TO SERINE ESTERASE INHIBITORS RESULTS IN THE INACTIVATION OF THE ESTERASE AND AN INCREASING LEVEL OF ESTER. THE INCREASE OF ESTER WOULD RESULT IN ACTIVATION OF THE RECEPTOR AND HISTAMINE RELEASE.

METHOD

Histamine Release: Histamine release was investigated on dextran-EDTA isolated human leukocytes following a 45 minute incubation at 37°C in the presence of esterase inhibitors unless indicated otherwise. For these studies, leukocytes derived from 2 ml of blood and suspended in 1 ml of Tyrode's buffer were used. After incubation, cells were centrifuged at 14,000G for one minute and the histamine content of 0.8 ml of the supernatant added to 0.2 ml of 10% perchloric acid was determined by the automated fluorometric method. Siraganian. 1974. Anal. Biochem. 57:383.

Nicotinamide Adenine Dinucleotide ($NAD^{\pm}$) Determinations: The cell pellet and the remaining 0.2 ml of supernatant were extracted overnight with 0.8 ml of 0.5 N perchloric acid and then neutralized with 0.2 ml of KOH After removal of the $KClO_4$ precipitate, NAD^+ determinations were performed by the method of Jacobson and Jacobson. NAD^+ levels were determined by an alcohol dehydrogenase cycling assay using 0.5 ml of leukocyte extract or appropriate NAD^+ standards. Samples were incubated at 37°C with the following materials in a final volume of 1.2 ml: 100 mM sodium bicine, pH 7.8; 500 mM ethanol; 0.42 mM MTT-tetrazolium, 1.66 mM phenazine ethosulfate, 4.16 mM EDTA; 0.83mg/ml of bovine serum albumin; and 0.1 ml of alcohol dehydrogenase (175 units/ml). After 30 minutes, the reaction was terminated

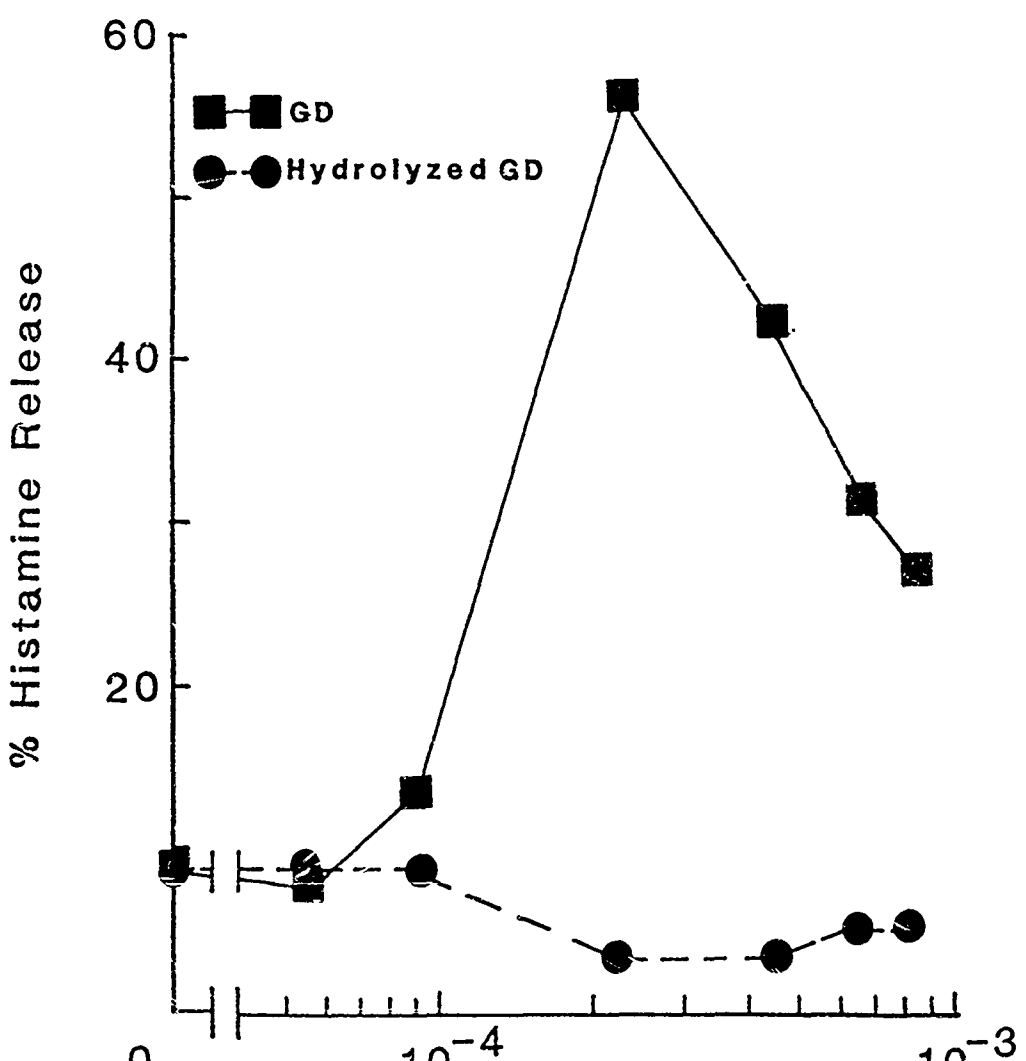

FIGURE 1: GD-DEPENDENT HISTAMINE RELEASE CAN BE PREVENTED BY HYDROLYSIS OF GD TO REMOVE THE FLUORINE. GD AT 10^{-2}M WAS INCUBATED FOR 8 HOURS AT 37^{o}C AT PH 12. GD AND PRE-HYDROLYZED GD WERE NEUTRALIZED WITH 0.05M HEPES IN TYRODE'S BUFFER TO A CONCENTRATION OF 10^{-3}M BEFORE INCUBATION WITH HUMAN LEUKOCYTES. THE PRE-HYDROLYZED GD FAILED TO RELEASE HISTAMINE WHILE THE HISTAMINE RELEASE OCCURRED AT THE NORMAL GD CONCENTRATION. EACH POINT IS THE AVERAGE OF THREE SEPARATE HISTAMINE DETERMINATIONS.

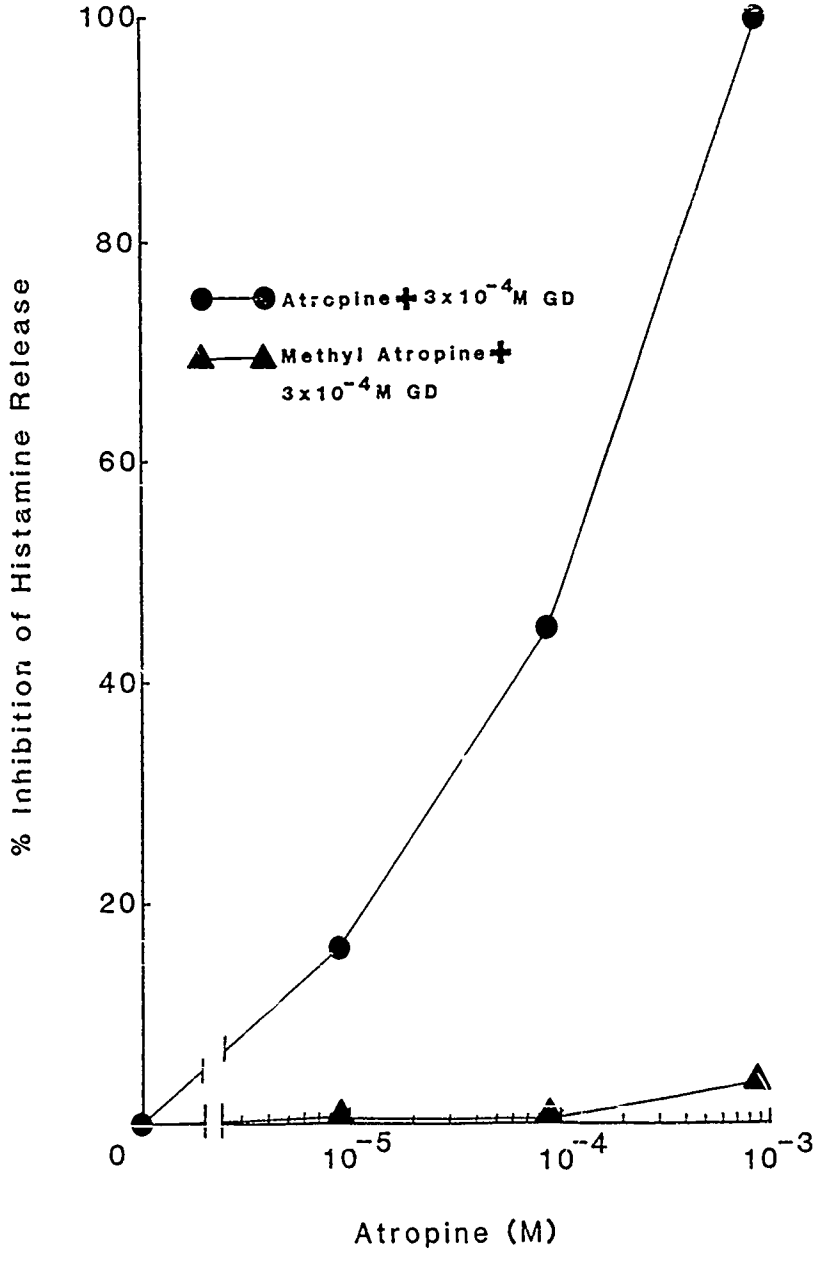

FIGURE 2 TO DETERMINE WHETHER ATROPINE WAS ACTING ON AN EXTRACELLULAR OR INTRACELLULAR MUSCARINIC-LIKE RECEPTOR, HUMAN LEUKOCYTES WERE PREINCUBATED FOR 15 MINUTES IN THE PRESENCE OF EITHER ATROPINE (AN UNCHARGED MUSCARINIC ANTAGONIST) OR METHYL ATROPINE (A POSITIVELY CHARGED MUSCARINIC ANTAGONIST) BEFORE EXPOSURE TO $3x10^{-4}$ M GD. ATROPINE AT $8x10^{-4}$ M TOTALLY INHIBITED HISTAMINE RELEASE BY GD AT 10^{-3} M AND BEGAN INHIBITING THIS RELEASE AT $9x10^{-6}$ M. HOWEVER, METHYL ATROPINE DID NOT CAUSE ANY SIGNIFICANT INHIBITION OF HISTAMINE RELEASE BY GD, EVEN AS HIGH AS $9x10^{-4}$ M. SINCE BOTH ATROPINE AND METHYL ATROPINE ARE KNOWN TO INHIBIT PERIPHERAL MUSCARINIC RECEPTORS, IT APPEARS THAT THE DISCREPANCY BETWEEN ATROPINE AND METHYL ATROPINE MAY BE THE RESULT OF METHYL ATROPINE'S INABILITY TO CROSS THE CELL MEMBRANE. THIS INDICATES THAT THE MUSCARINIC-LIKE RECEPTOR MUST BE INTRACELLULAR. EACH POINT IS THE AVERAGE OF THREE SEPARATE HISTAMINE DETERMINATIONS.

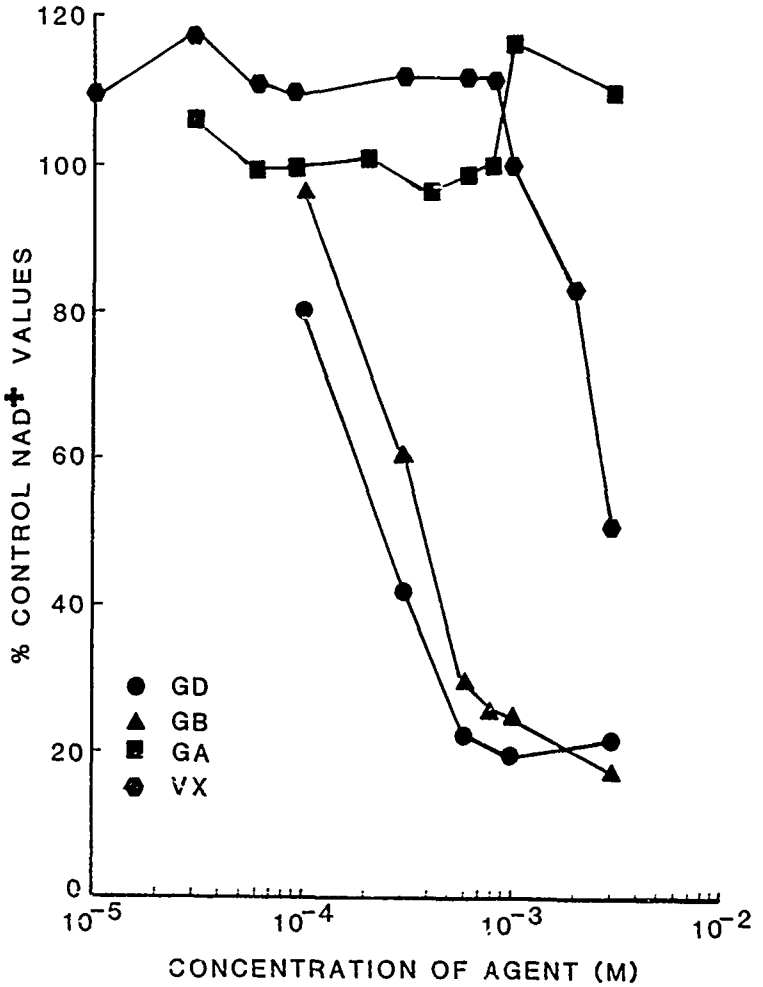

FIGURE 3: THE ABILITY OF GD TO LOWER NAD^+ LEVELS OF HUMAN LEUKOCYTES WAS COMPARED TO OTHER ACETYLCHOLINESTERASE INHIBITORS. GB, ANOTHER ORGANOPHOSPHONATE, SHOWED A SIMILAR DOSE RESPONSE CURVE TO GD IN LOWERING NAD^+. VX, AN ORGANOPHOSPHONOTHIOLATE, ALSO LOWERED NAD^+ LEVELS OF THE LEUKOCYTES, BUT REQUIRED VERY HIGH CONCENTRATIONS TO ACHIEVE A 50% DROP IN NAD^+ LEVELS. GA, AN ORGANOPHOSPHORAMIDOCYANIDATE, FAILED TO CAUSE A DECREASE IN NAD^+ LEVELS AT DOSES AS HIGH AS 10^{-2}M EVEN THOUGH IT IS A COMPARABLE INHIBITOR OF ACETYLCHOLINESTERASE AND CARBOXYESTERASES. THE INCUBATION WAS FOR 1 HOUR AT 37°C. ALL POINTS REPRESENT THE AVERAGE OF THREE SAMPLES WHICH WERE ASSAYED IN DUPLICATE FOR NAD^+.

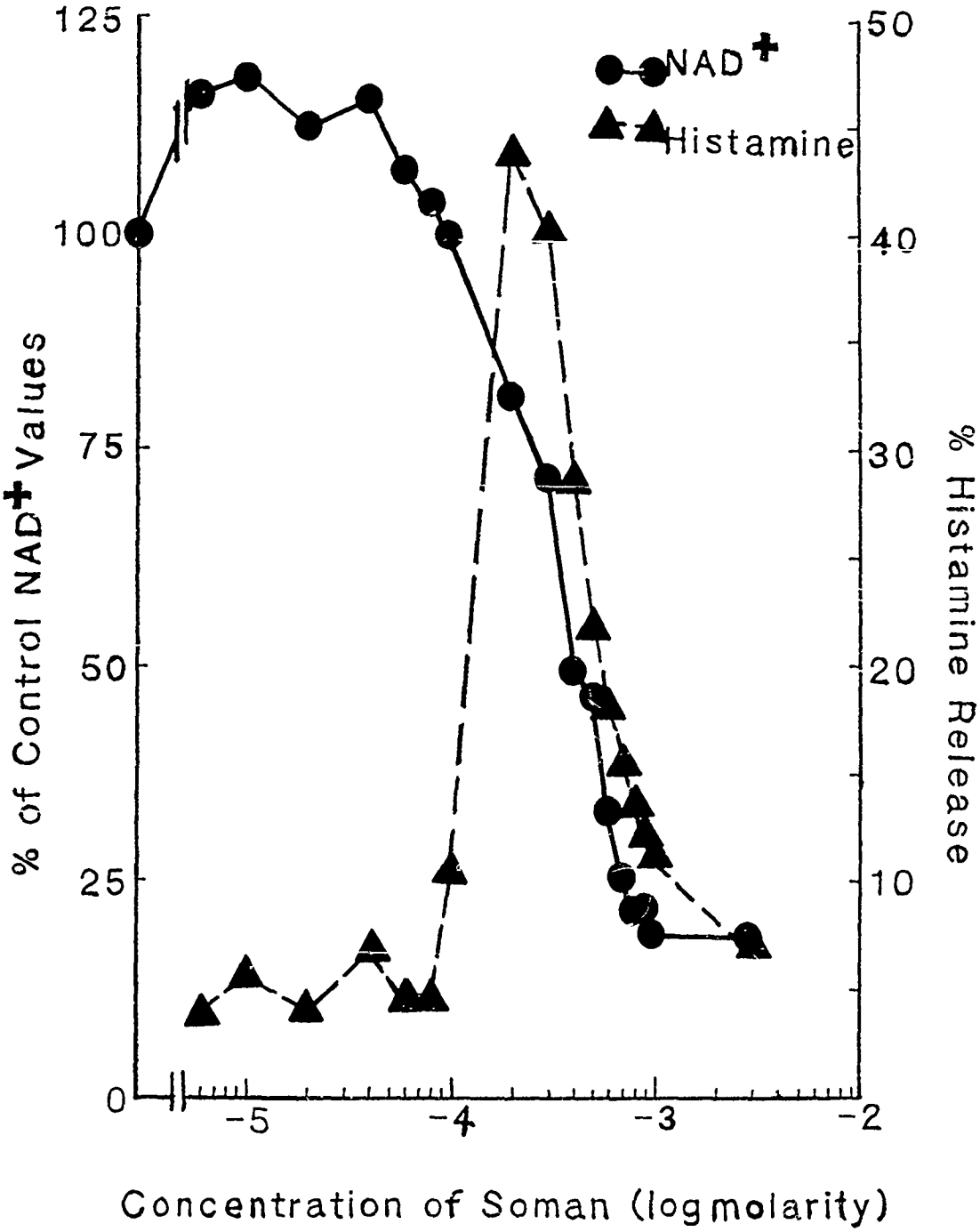

FIGURE 4: THIS FIGURE DEMONSTRATES THAT AT A CONCENTRATION OF THE INHIBITOR THAT LOWERS NAD^+ BY ABOUT 50%, THE ABILITY OF THIS INHIBITOR TO RELEASE HISTAMINE BEGINS TO DECREASE. THE INCUBATION WAS FOR 1 HOUR AT $37^{o}C$. ALL POINTS REPRESENT THE AVERAGE OF THREE SAMPLES WHICH WERE ASSAYED IN DUPLICATE FOR NAD^+; THE HISTAMINE RELEASE WAS DETERMINED SPECTROPHOTOMETRICALLY.

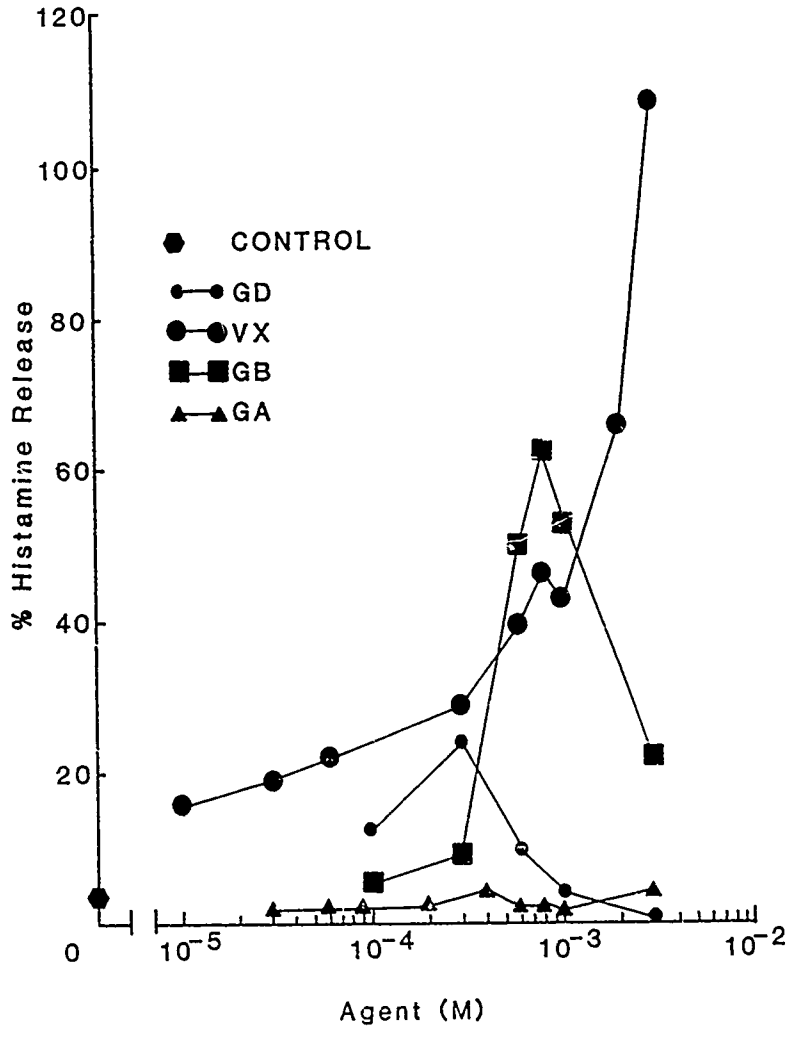

FIGURE 5 HUMAN LEUKOCYTES WERE EXPOSED TO A LARGE VARIETY OF ESTERASE INHIBITORS TO DETERMINE WHAT KIND OF ESTERASE WAS BEING INACTIVATED BY GD AND DFP. VX, A HIGHLY SPECIFIC ACETYLCHOLINESTERASE INHIBITOR, WAS REQUIRED AT MUCH HIGHER CONCENTRATIONS THAN GD TO RELEASE HISTAMINE. GA, AN EFFECTIVE INHIBITOR OF ALIESTERASES (OOMS AND BREEBAART-HANSEN, BIOCHEM. PHARM. 1965. 14:1727) AS WELL AS ACETYLCHOLINESTERASE, FAILED TO CAUSE HISTAMINE RELEASE FROM HUMAN LEUKOCYTES. GB, ANOTHER ORGANOPHOSPHONATE, SHOWED SIMILAR HISTAMINE RELEASING PROPERTIES TO GD. EACH POINT IS THE AVERAGE OF THREE SEPARATE HISTAMINE DETERMINATIONS.

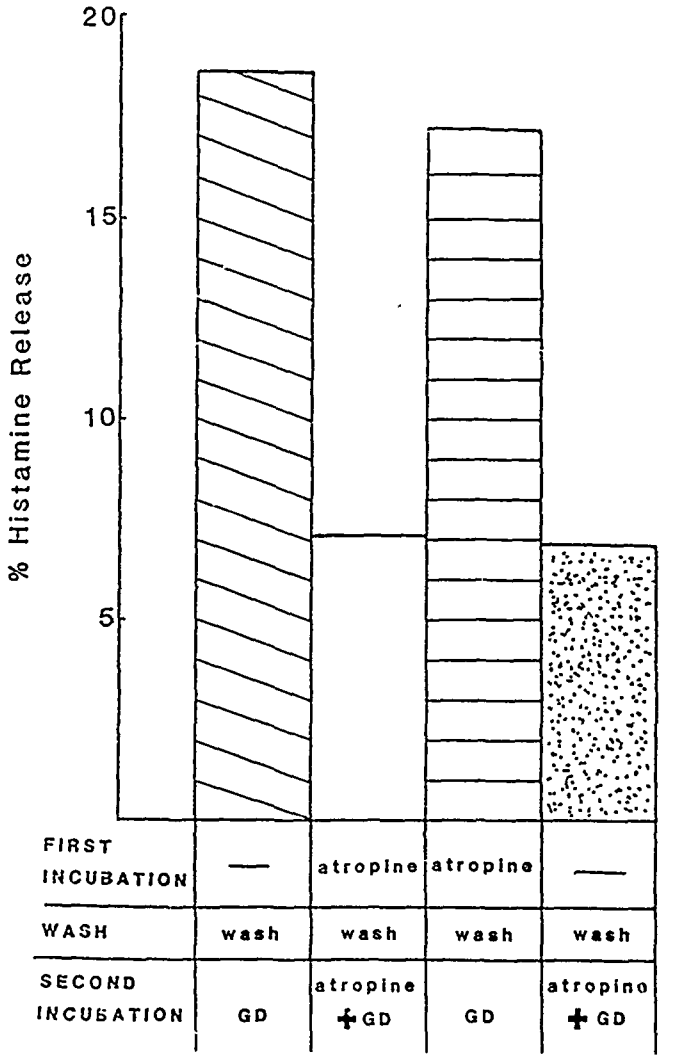

FIGURE 6: IT APPEARS THAT INHIBITION OF GD-DEPENDENT HISTAMINE RELEASE IS REVERSIBLE. CELLS WERE INCUBATED FOR 30 MINUTES AT 37°C WITH BUFFER OR 8×10^{-4}M ATROPINE. AFTER THE INITIAL INCUBATION, CELLS WERE WASHED TWICE WITH BUFFER AND THEN CHALLENGED FOR 30 MINUTES AT 37°C WITH GD OR GD AND ATROPINE. EACH POINT IS THE AVERAGE OF THREE SEPARATE HISTAMINE DETERMINATIONS.

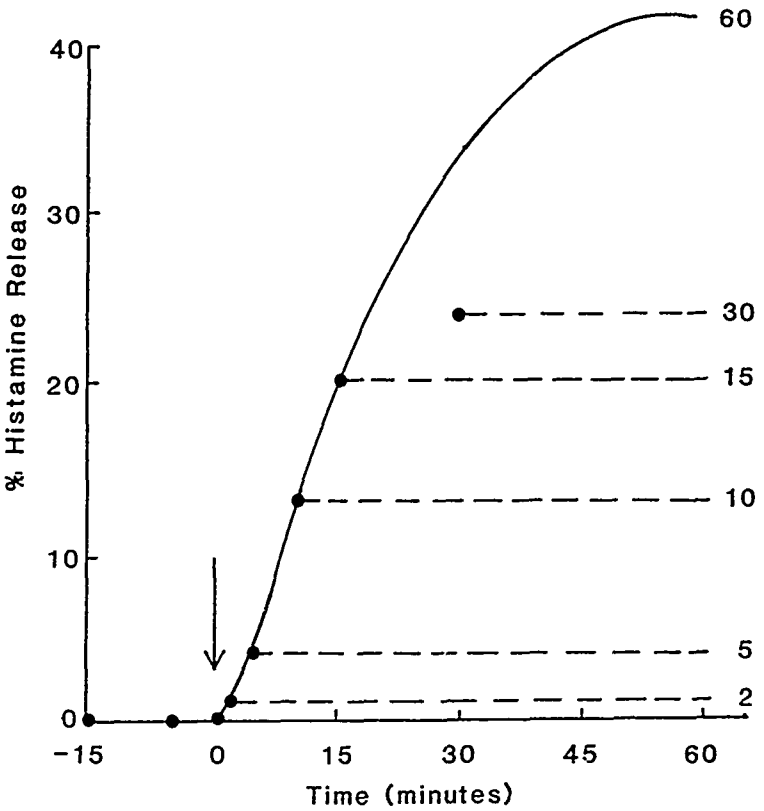

FIGURE 7 HISTAMINE RELEASE BY ESTERASE INHIBITORS COULD BE BLOCKED BY PRE-INCUBATION WITH ATROPINE. HUMAN LEUKOCYTES WERE INCUBATED FOR 75 MINUTES AT 37° C. GD WAS ADDED 15 MINUTES AFTER THE INCUBATION WAS INITIATED (TIME 0). ATROPINE, 8×10^{-4} M, WAS ADDED AT THE INDICATED TIMES. ATROPINE ADDED AS LATE AS 15 MINUTES AFTER GD STILL RESULTED IN 50% INHIBITION OF HISTAMINE RELEASE. ATROPINE ADDED CONCURRENTLY WITH GD (TIME 0) RESULTED IN COMPLETE INHIBITION OF HISTAMINE RELEASE. EACH POINT IS THE AVERAGE OF THREE SEPARATE HISTAMINE DETERMINATIONS.

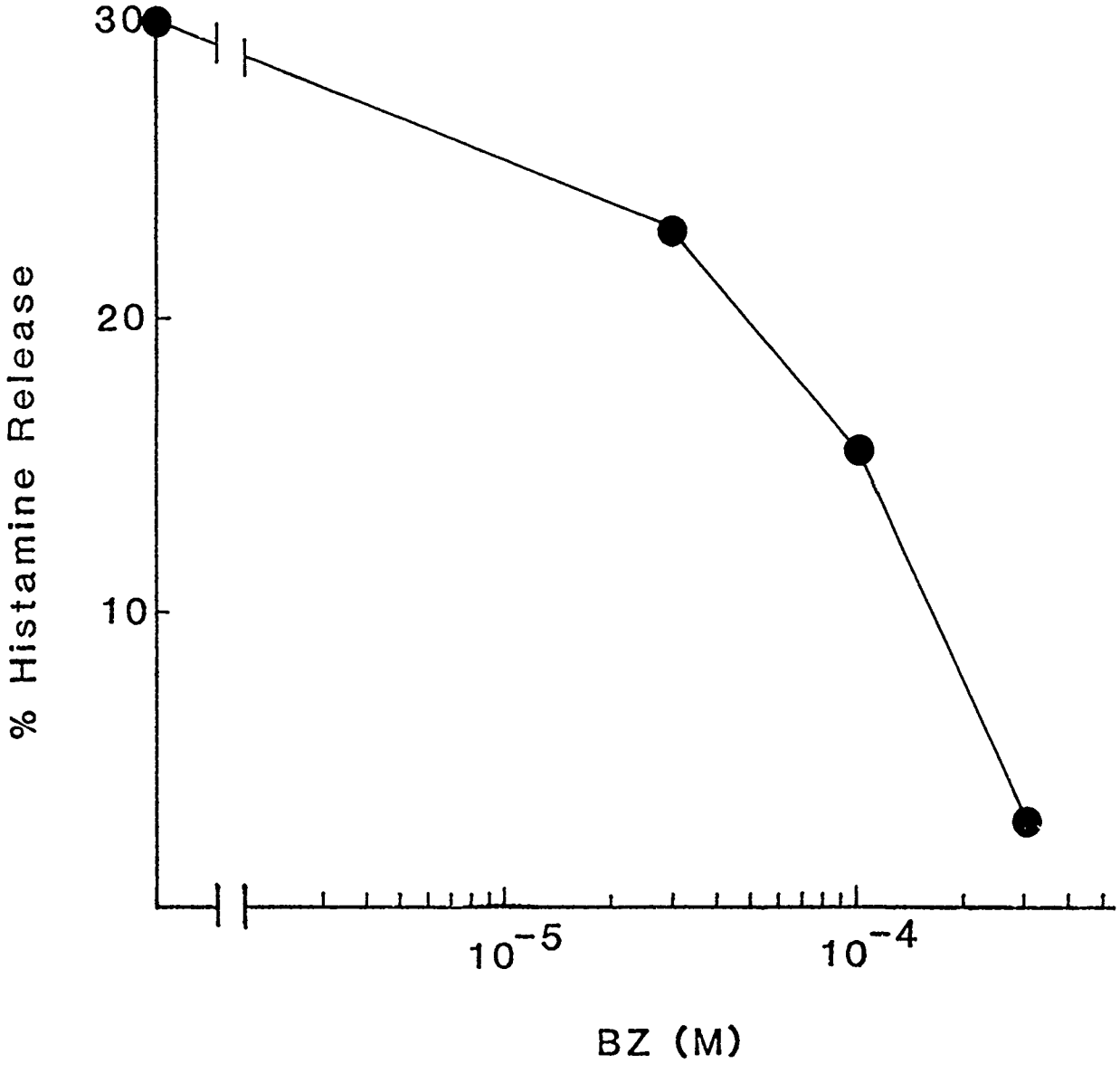

FIGURE 8 3-QUINUCLIDINYL BENZILATE (BZ) IS A MORE POTENT MUSCARINIC ANTAGONIST THAN ATROPINE AND ALSO APPEARS TO BE MORE EFFECTIVE AT INHIBITING ESTERASE-SPECIFIC HISTAMINE RELEASE. BZ CAUSED A 50% INHIBITION OF GD-DEPENDENT HISTAMINE RELEASE AT 10^{-4}M. THE RESULTS USING BZ INDICATE THAT INHIBITION OF GD-DEPENDENT HISTAMINE RELEASE WAS NOT SPECIFIC FOR ATROPINE BUT APPEAR TO BE A PROPERTY OF THIS TYPE OF MUSCARINIC ANTAGONIST. THESE RESULT STRENGTHEN THE ARGUMENT THAT INHIBITION OF HISTAMINE RELEASE IS BY A MUSCARINIC-LIKE RECEPTOR. EACH POINT IS THE AVERAGE OF THREE SEPARATE HISTAMINE DETERMINATIONS.

AGENT	SOMAN GD pinacolyl methyl phosphonofluoridate	SARIN GB isopropyl methylphosphonofluoridate	TABUN GA ethyl N,N-dimethylphosphoramidocyanidate	VX O-ethyl S-(2-diisopropylaminoethyl) methyl phosphonothiolate	DFP diisopropyl fluorophosphate
PEAK HISTAMINE RELEASE	3×10^{-4}M	8×10^{-4}M	FAILED TO CAUSE HISTAMINE RELEASE	3×10^{-3}M	10^{-3}M
50% NAD^+ DECREASE	4×10^{-4}M	6×10^{-4}M	FAILED TO CAUSE NAD^+ DECREASE	3×10^{-3}M	3×10^{-3}M

CONCLUSIONS

A

1. HISTAMINE IS RELEASED FROM HUMAN BASOPHILS BY ORGANOPHOSPHOROUS INHIBITORS OF ACETYLCHOLINESTERASE.

2. THESE INHIBITORS APPEAR TO BE ACTING INDEPENDENTLY OF THE ACETYLCHOLINE-ACETYLCHOLINESTERASE SYSTEM.

3. THE INHIBITORS WITH HIGH AFFINITY FOR ALIESTERASES FAILED TO RELEASE HISTAMINE BY THEMSELVES AND PREVENTED THE RELEASE OF HISTAMINE BY GD.

CONCLUSIONS

B

1. ANTAGONISTS FOR MUSCARINIC SITES CAUSE A DOSE-DEPENDENT INHIBITION OF ESTERASE INHIBITOR-INITIATED HISTAMINE RELEASE.

2. ONLY NEUTRAL MUSCARINIC ANTAGONISTS AFFECT HISTAMINE RELEASE, INDICATING THAT THE RECEPTOR IS INTRACELLULAR.

3. THE ANTAGONISTS REACT TO IMMEDIATELY BLOCK GD-DEPENDENT HISTAMINE RELEASE AND THE BLOCKAGE IS REVERSIBLE.

CONCLUSIONS

C

1. ORGANOPHOSPHOROUS FLUORINE ACETYL-CHOLINESTERASE INHIBITORS CAUSE A DOSE-DEPENDENT DECREASE IN NAD^+ LEVELS.

2. THE DECREASE IN NAD^+ LEVELS BY THESE INHIBITORS APPEARS TO BE INDEPENDENT OF THE ACETYLCHOLINE-ACETYLCHOLINESTERASE SYSTEM.

3. THE EFFECT OF THESE INHIBITORS DOES NOT INVOLVE A MUSCARINIC-LIKE RECEPTOR.

4. AT A CONCENTRATION OF THE INHIBITOR THAT LOWERS NAD^+ BY ABOUT 50%, THE ABILITY OF THIS INHIBITOR TO RELEASE HISTAMINE BEGINS TO DECREASE.

LONG-TERM EFFECTS OF THE ORGANOPHOSPHATE DFP ON HORMONAL RHYTHMS IN THE RAT

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(Spon: G.R. Sessions)
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ORGANOPHOSPHATE EXPOSURE HAS BEEN REPORTED TO CAUSE LONG TERM BEHAVIORAL EFFECTS IN HUMANS (EEG CHANGES, INSOMNIA) AND IN EXPERIMENTAL ANIMALS.

WE HAVE DEMONSTRATED IN OUR LABORATORY THAT A SINGLE INJECTION OF THE IRREVERSIBLE CHOLINESTERASE INHIBITOR DIISOPROPYLFLUORO-PHOSPHATE (DFP) DISRUPTS NORMAL CIRCADIAN EATING PATTERNS AND OTHER NORMAL ACTIVITY IN RATS FOR SEVERAL WEEKS.

WE HAVE ALSO REPORTED THAT A SINGLE INJECTION OF THE CHOLINERGIC AGONISTS PHYSOSTIGMINE, NEOSTIGMINE, OXOTREMORINE AND NICOTINE PRODUCES AN IMMEDIATE AND PRONOUNCED INCREASE IN PLASMA LEVELS OF β-ENDORPHIN (β-EP), β-LIPOTROPHIN (β-LPH), CORTICOSTERONE (CS) AND PROLACTIN (PRL) IN RATS.

SINCE THESE HORMONES ARE KNOWN TO EXHIBIT CIRCADIAN RHYTHMS WE UNDERTOOK A STUDY OF THE EFFECTS OF DFP EXPOSURE (SINGLE INJECTION) ON LONG TERM CIRCADIAN HORMONAL PATTERNS AND WHETHER ANTIDOTE PRETREATMENT MIGHT ALTER ANY SUCH EFFECTS.

METHODS

NAIVE MALE SPRAGUE-DAWLEY RATS (300 ± 25 G) WERE INDIVIDUALLY CAGED IN A ROOM WHICH WAS ON A CONTROLLED LIGHT/DARK CYCLE (0600-1800 LIGHT/1800-0600 DARK).

THE ANIMALS WERE DIVIDED INTO THREE TREATMENT GROUPS:

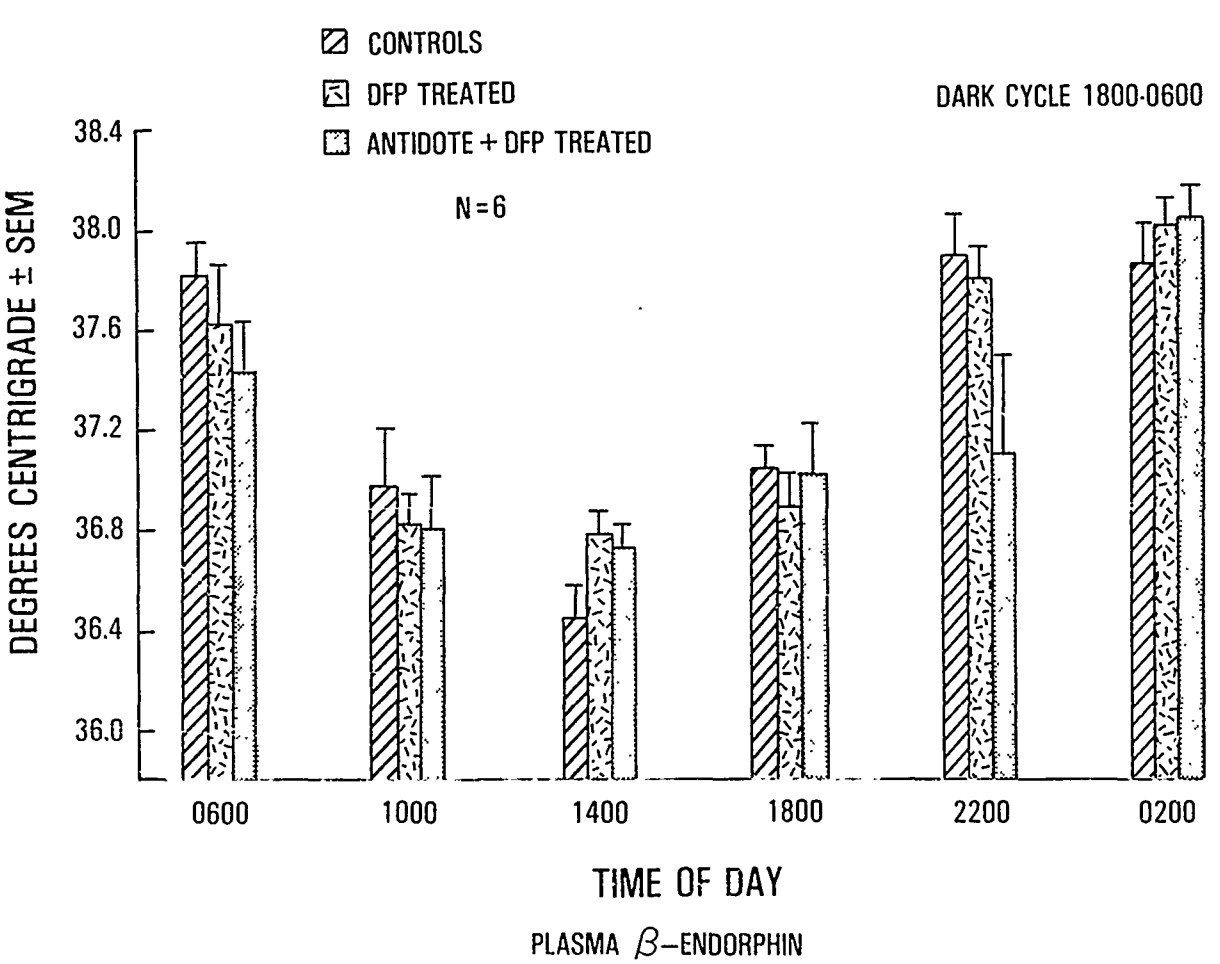
1. SALINE INJECTION
2. DFP (2.6MG/KG) INJECTION
3. ATROPINE SULFATE (25MG/KG) PLUS 2-PAM CHLORIDE (12.5MG/KG) FOLLOWED 15 MIN LATER BY DFP (2.6MG/KG) INJECTION

ALL INJECTIONS WERE GIVEN IP. THE ANIMALS RECEIVING DFP ALONE SHOWED A 35% MORTALITY RATE.

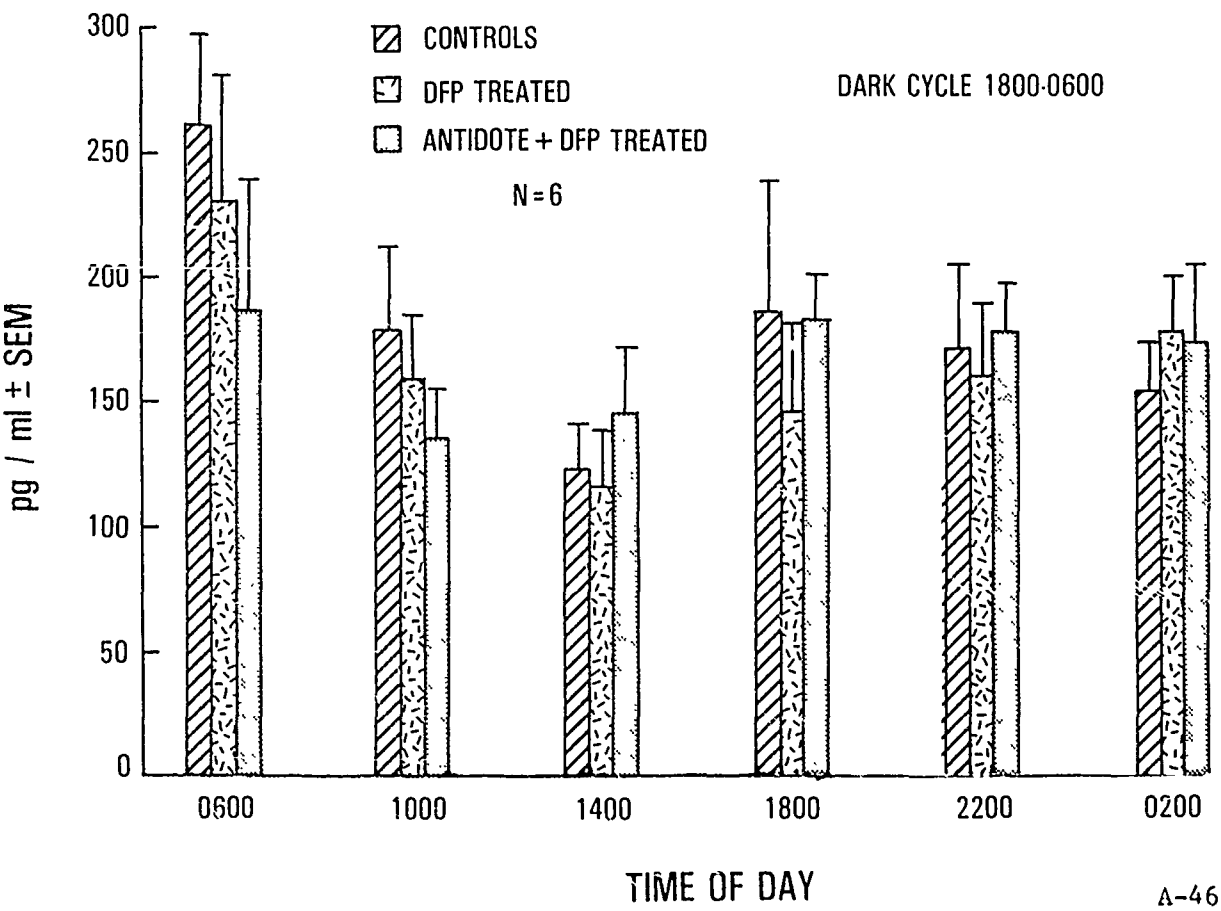
TWO WEEKS FOLLOWING THE INJECTIONS, 18 ANIMALS (6 FROM EACH TREATMENT GROUP) WERE SACRIFICED BY DECAPITATION EVERY FOUR HOURS STARTING AT 0600 HOURS. RECTAL TEMPERATURE MEASUREMENTS WERE TAKEN IMMEDIATELY FOLLOWING BLOOD COLLECTION.

TRUNK BLOOD WAS COLLECTED IN HEPARINIZED TUBES AND CENTRIFUGED FOR 15 MIN AT 2500 RPM (4°C). TWO ML OF THE PLASMA WAS TRANSFERRED TO A SEPARATE TUBE CONTAINING 50 µL (.90 TIU) OF TRASYLOL FOR ASSAY OF β-EP AND β-LPH. THE REMAINDER OF THE PLASMA WAS TRANSFERRED TO A SEPARATE TUBE FOR CS AND PRL ASSAY.

BODY TEMPERATURE



PLASMA β–ENDORPHIN



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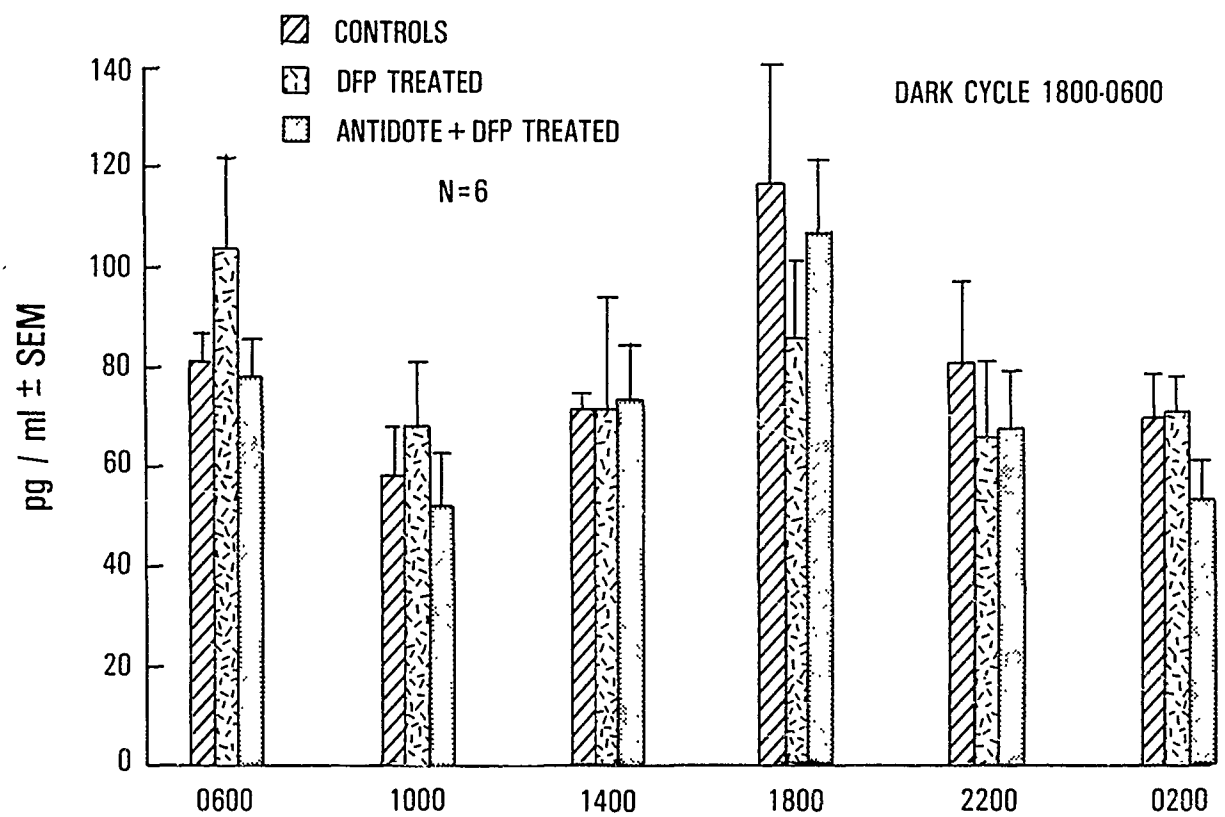
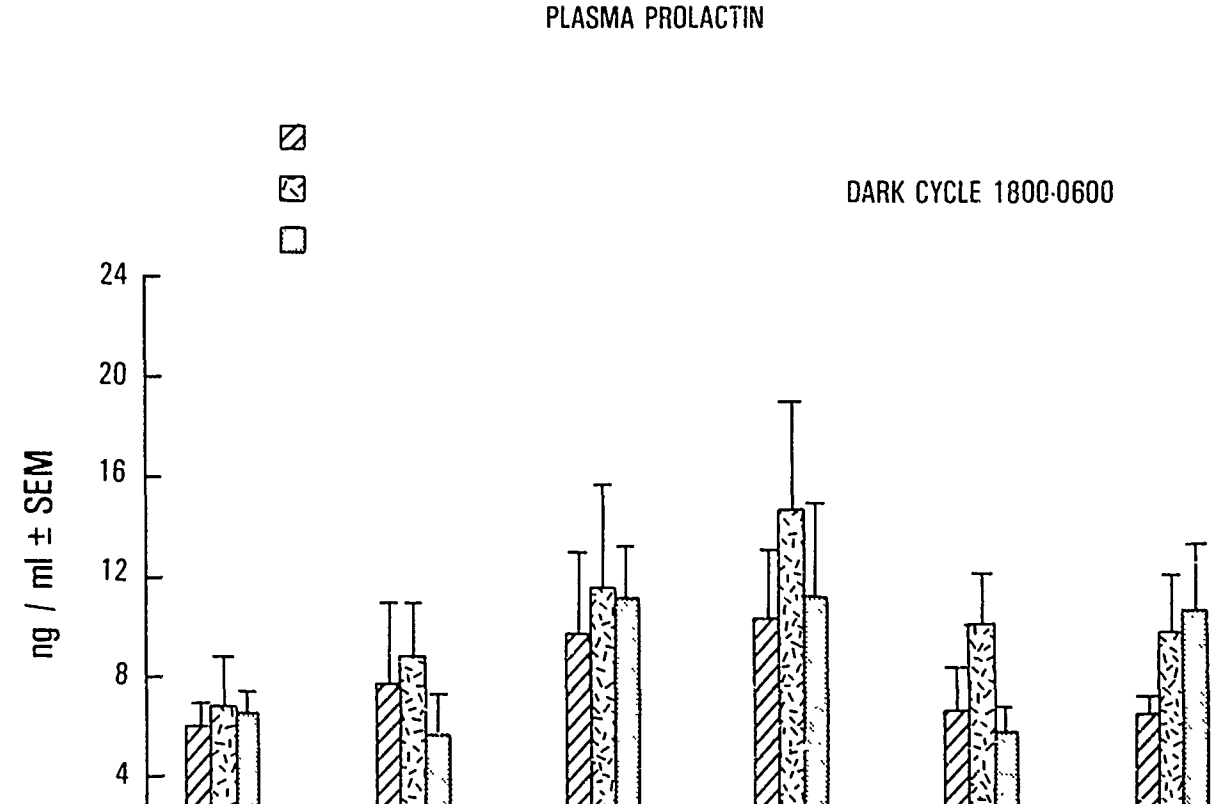
PLASMA β-LPH



PLASMA PROLACTIN



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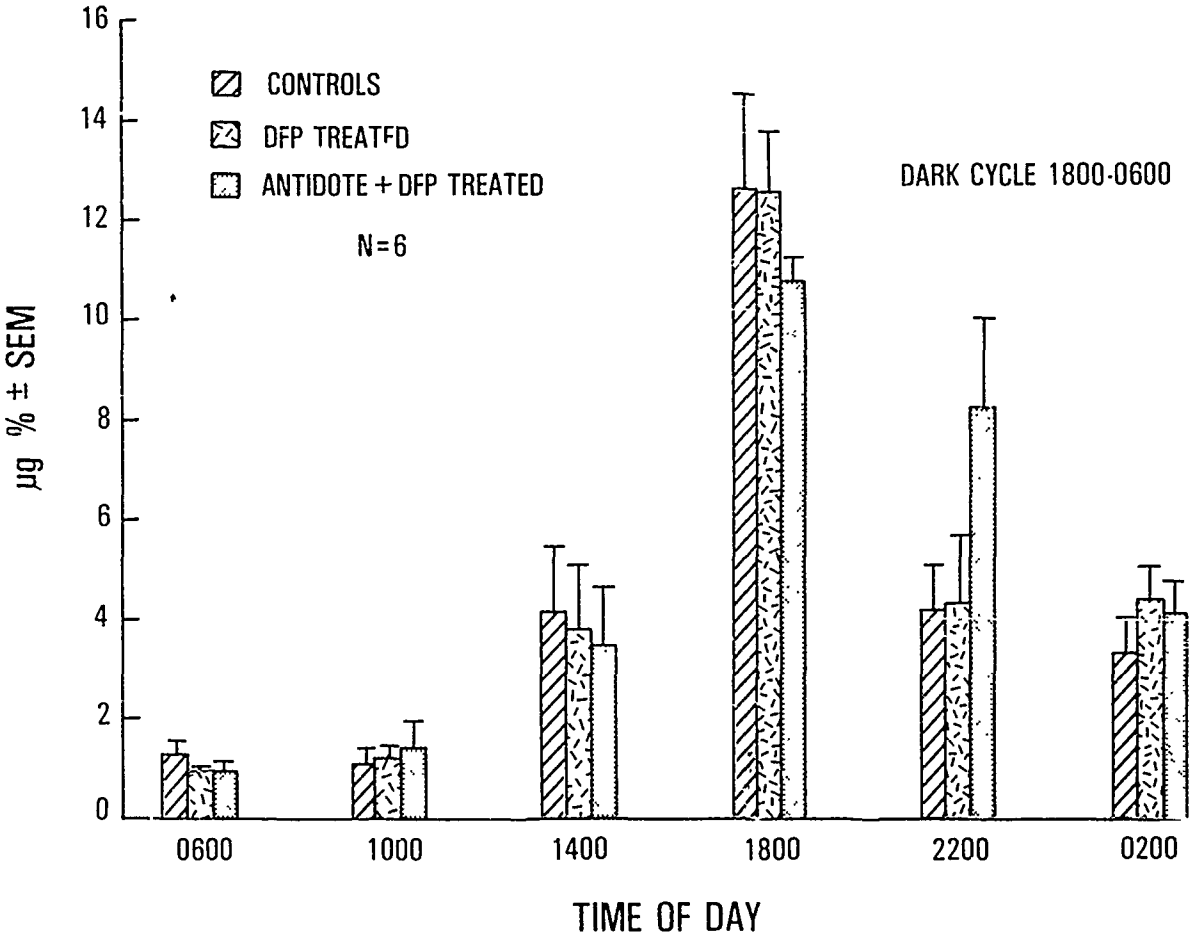
PLASMA CORTICOSTERONE



CONCLUSIONS

LONG TERM DISRUPTIONS IN NORMAL HORMONAL PATTERNS AS A RESULT OF EXPOSURE TO THE ORGANOPHOSPHATE DFP WERE NOT FOUND. THERE WERE NO SIGNIFICANT DIFFERENCES AMONG THE THREE TREATMENT GROUPS.

BODY TEMPERATURES FOLLOWED THE REPORTED DIURNAL RHYTHM WITH A NADIR AT 1400 HRS AND THE HIGHEST VALUES RECORDED AT 0200-0600 HRS. PLASMA β-ENDORPHIN LEVELS APPEARED TO FOLLOW THIS SAME PATTERN.

PEAK LEVELS FOR CORTICOSTERONE, PROLACTIN AND β-LIPOTROPHIN WERE SEEN AT 1800 HRS. THE LOWEST VALUES FOR BOTH CORTICOSTERONE AND PROLACTIN WERE SEEN AT 0600 HRS, WHEREAS THE LOWEST VALUES FOR β-LIPOTROPHIN WERE FOUND AT 1000HRS.

PENTOBARBITAL ANESTHESIA IN ORGANOPHOSPHATE INTOXICATION IN NONHUMAN PRIMATES

W.G. Johanson, Jr., A. Anzueto, G.G. Berdine, G.T. Moore and C.D. White
Southwest Research Institute, University of Texas Health Science Center
Southwest Foundation for Biomedical Research, and
Audie Murphy Veterans Administration Hospital, San Antonio, Texas

ABSTRACT

It is known that a number of drugs affect the metabolism of organophosphate compounds or otherwise affect the manifestations of intoxication by these agents. For example, the toxicity of Soman (pinacolyl methylphosphonofluoridate) in mice is reduced by pretreatment with sodium phenobarbital (Biochem. Pharmacol. 32:1411, 1983) and is potentiated by pentobarbital pretreatment (Biochem. Pharmacol. 33:683, 1984). These observations have important implications for the interpretation of experimental intoxications in which animals are anesthetized, for management of human casualties, and for the development of strategies to prevent death or disability in exposed humans.

To determine the effects of pentobarbital anesthesia in Soman intoxication in nonhuman primates, we studied 13 baboons (*Papio* sp.) weighing 11-27 kg. Soman, 13.1 μg/kg (2 x LD_{50}), was infused over 10 min via a peripheral vein. Animals were instrumented with arterial and pulmonary arterial catheters and ECG electrodes. In Group A (7 animals), animals were anesthetized with pentobarbital, 5 mg/kg, IV, and instrumented just prior to infusion. In Group B, instrumentation was performed 3 days prior to infusion with a tether system which allowed virtually unrestrained activity within the cage. Soman was administered without premedication. In both groups, ventilation was provided by high frequency jet ventilation (HFJV) via a cricothyroid cannula (CTC) when apnea ensued, and atropine, 3 mg IM, was administered following the onset of cardiovascular effects.

Measurements included mean blood pressure (MBP), cardiac output by thermodilution (CO), and heart rate (HR); cardiac index (CI) and systemic vascular resistance (SVR) were calculated.

ABSTRACT *(CONTINUED)*

In both groups, Soman produced copious secretions and apnea at an average time of 8 min of infusion. Mild muscle fasciculations were noticed transiently in Group A animals. Group B animals experienced severe muscle fasciculations that began at 4 min of infusion and remained for 3 or 4 hr. This muscle activity was associated with a severe lactic acidosis which was not observed in Group A.

Soman produced a decline in MBP, CO, HR, CI and SVR lasting 4 hr in Group A. Atropine rapidly increased HR and improved MBP and CO. In Group B, the decrease in MBP was not as severe as in Group A. Cardiac arrhythmias and severe bradycardia were swiftly improved with atropine in both groups. The hypotensive effect of Soman appears to be due principally to a loss of vascular tone and bradycardia, changes which were more severe in Group A.

Recovery was delayed in Group B animals. By 4 hr, three of the seven Group A animals had regained adequate spontaneous ventilation whereas all Group B animals remained apneic, although MBP and blood pH had nearly returned to baseline values in the latter.

In the unanesthetized state, Soman causes severe muscular fasciculations, severe metabolic acidosis, prolonged apnea and a mild decrease in blood pressure. Pentobarbital anesthesia ameliorates the neuromuscular effects and acidosis but prolongs and potentiates the hemodynamic effects of Soman. (This work was supported in part by the U.S. Army Medical Research and Development Command under Contract DAMD-17-83-CI-3080.)

ANIMAL GROUPS

Pentobarbital (seven animals)

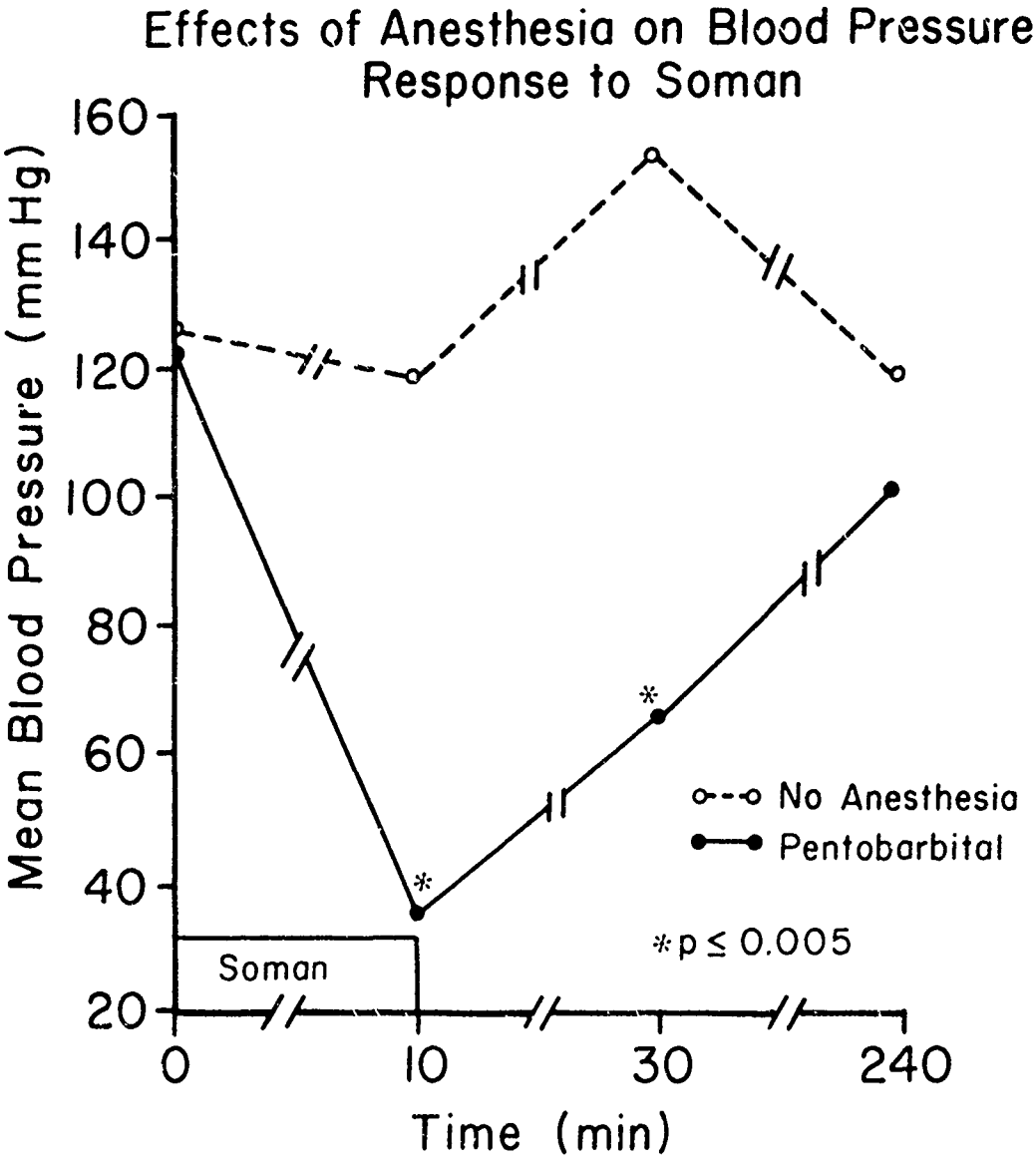
- — Anesthetized pentobarbital (5 mg/kg)
- — Instrumentation prior to infusion

No Anesthesia (six animals)

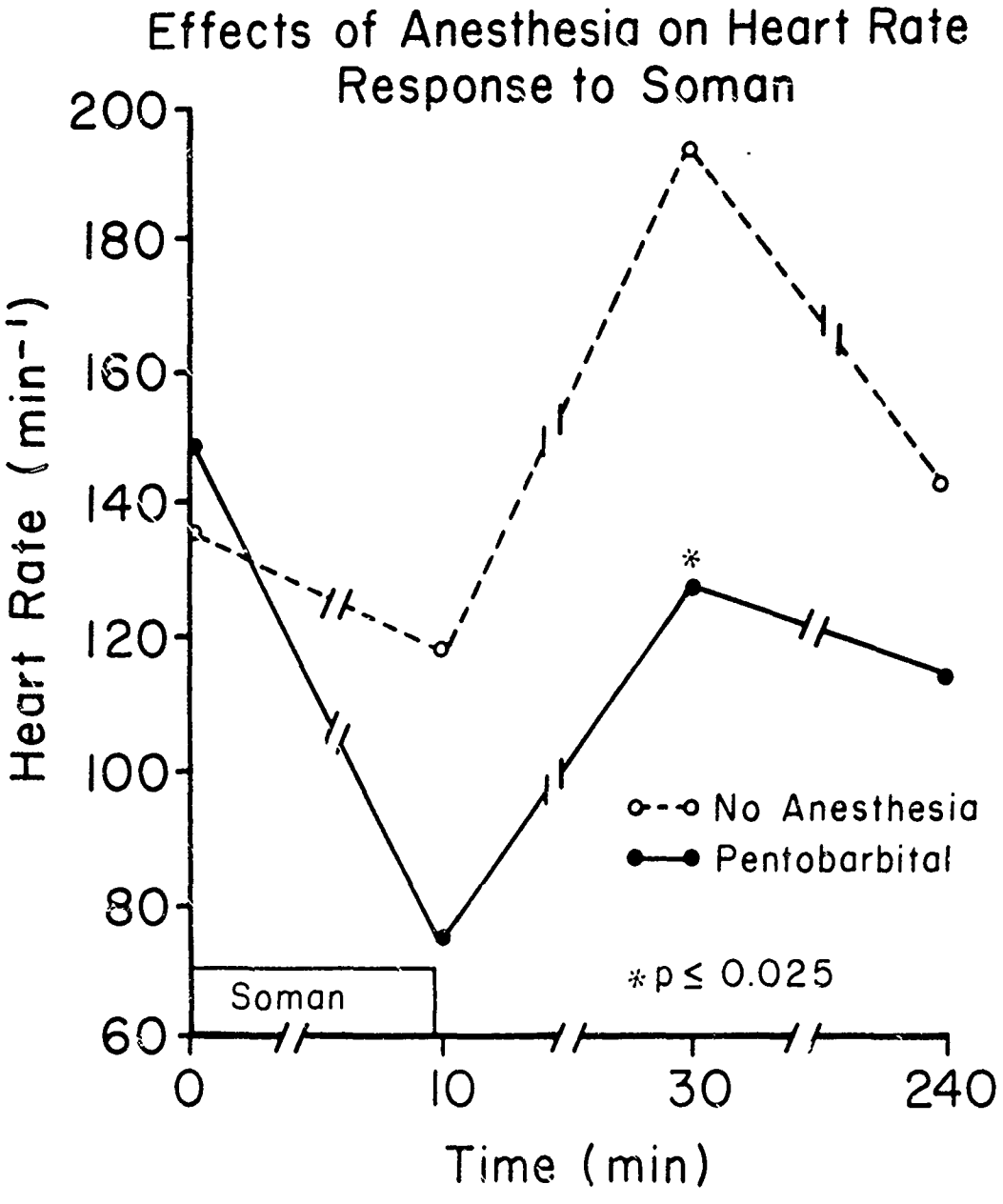
- — Instrumentation with a tether system 3 days prior to infusion
- — Infusion unrestrained

PROTOCOL

- — Animal model: baboon (*Papio* sp.), 11-27 kg.
- — Soman 13.1 μg/kg (2 x LD_{50}) IV, infused over 10 min via peripheral vein.
- — Animal instrumentation: arterial lines, pulmonary arterial catheter (Swan-Ganz) and ECG.
- — Measurements: blood pressure, cardiac output, heart rate, ECG, arterial blood gases.
- — Treatment: after Soman intoxication:
  - — HFJV 100% O_2 via cricothyroid cannula.
  - — Atropine (3 mg) IM.

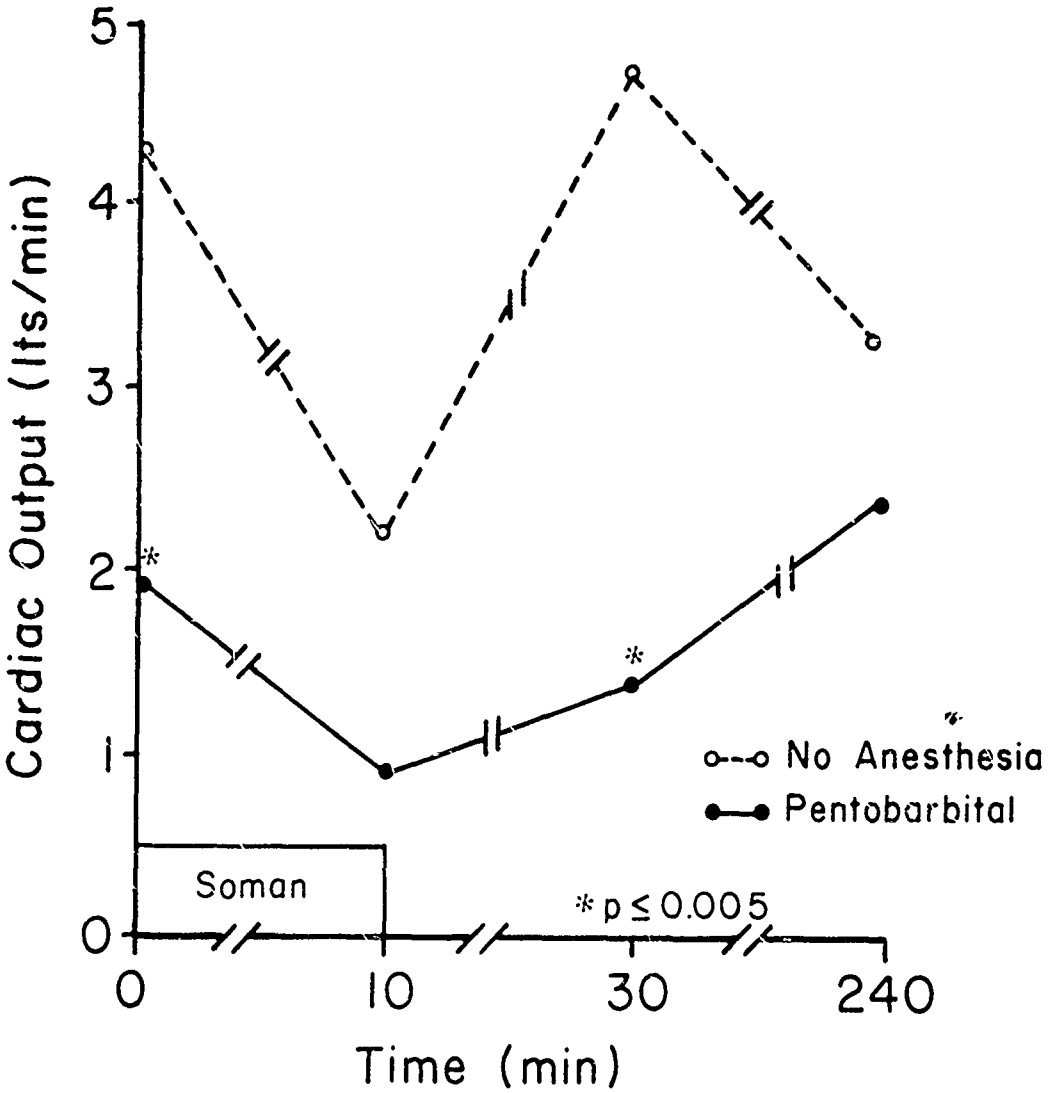


Effects of pentobarbital anesthesia on blood pressure response to Soman. Solid line (●—●) represents average values of seven animals exposed to Soman during anesthesia with pentobarbital. Dotted lines (o--o) represent average values of six tethered, unanesthetized animals exposed to Soman. Soman (13.14 μg/kg) was infused over a 10 min period. Both groups received atropine (3 mg total dose) injected within the infusion period.



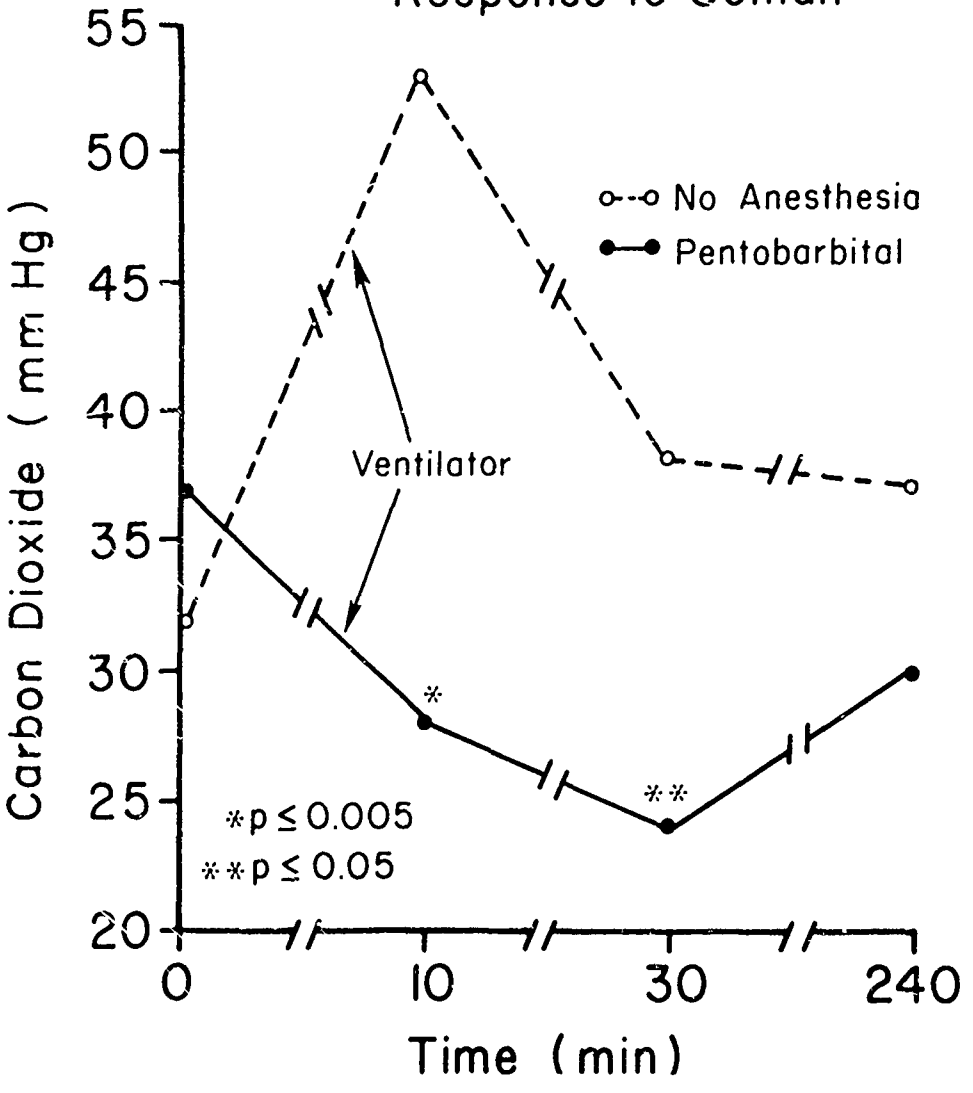
Effects of pentobarbital anesthesia on heart rate response to Soman. Soman (13.14 μg/kg) was infused over a 10 min period. Solid line (●—●) represents average values of seven exposed animals anesthetized with sodium pentobarbital. Dotted lines (o--o) represent average values of six tethered, unanesthetized exposed animals. Both groups received atropine (3 mg total dose) injected within the infusion period.

Effects of Anesthesia on Cardiac Output Response to Soman

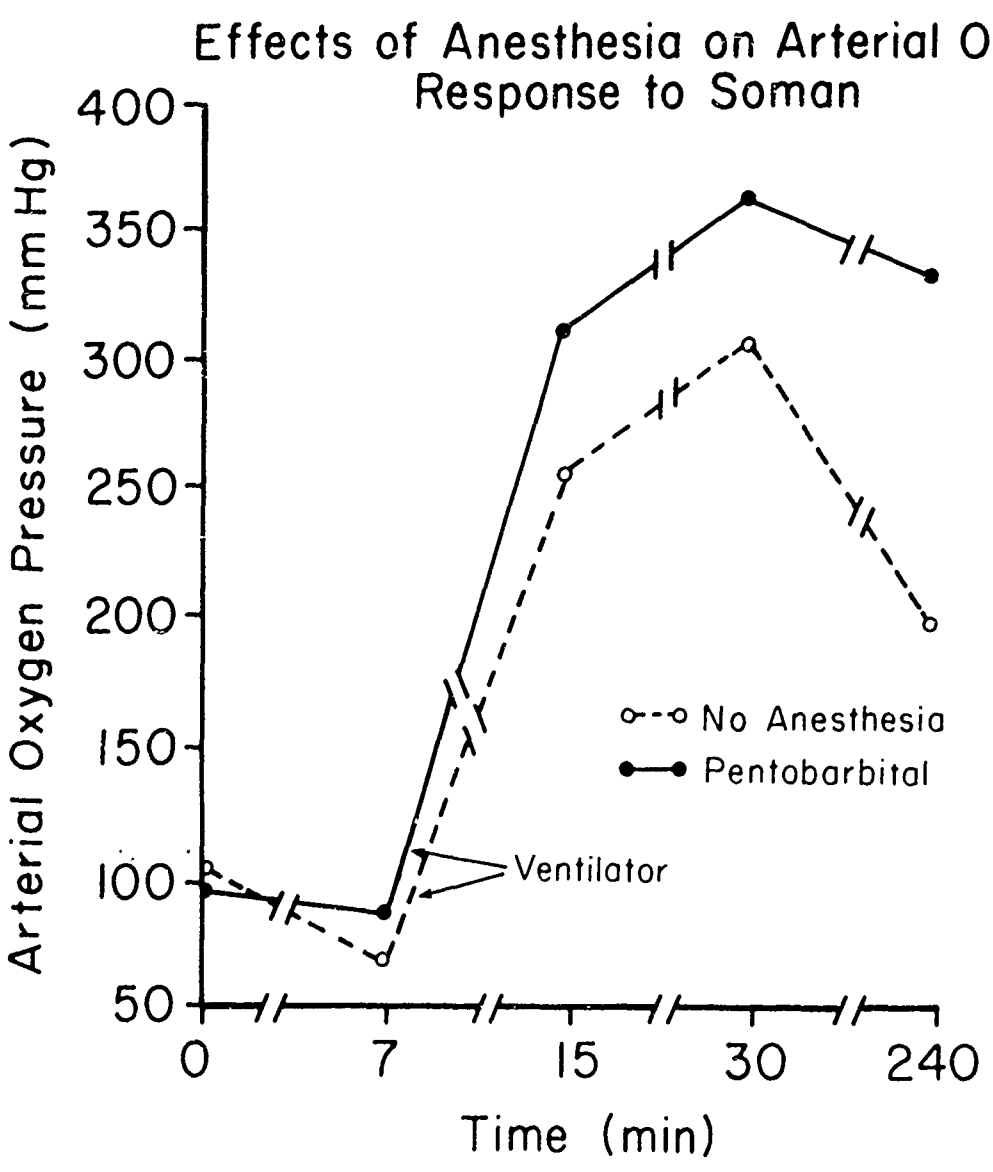


Effects of pentobarbital anesthesia on thermodilution cardiac output response to Soman. Soman (13.14 μg/kg) was infused over a 10 min period. Solid line (●—●) represents average values of seven exposed animals anesthetized with sodium pentobarbital. Dotted lines (o--o) represent average values of six tethered, unanesthetized exposed animals. Both groups received atropine (3 mg total dose) injected within the infusion period.

Effects of Anesthesia on Carbon Dioxide Response to Soman



Effects of pentobarbital anesthesia on carbon dioxide tension ($PaCO_2$) response to Soman. Solid line (●—●) represents average values of seven exposed animals anesthetized. Dotted line (o--o) represents average values of six tethered, unanesthetized exposed animals. Soman (13.14 μg/kg) was infused over a 10 min period. Animals were ventilated by HFJV, 100% O_2 via CTC. Both groups received atropine (3 mg total dose) injected within the infusion period.



Effects of pentobarbital anesthesia on arterial oxygen tension (PaO_2) response to Soman. Solid line (●—●) represents average values of seven animals anesthetized with sodium pentobarbital. Dotted lines (o--o) represent average values of six tethered, unanesthetized exposed animals. Soman (13.14 μg/kg) was infused over a 10 min period. Animals were ventilated by HFJV, 100% O_2 via CTC. Both groups received atropine (3 mg total dose) injected within the infusion period.

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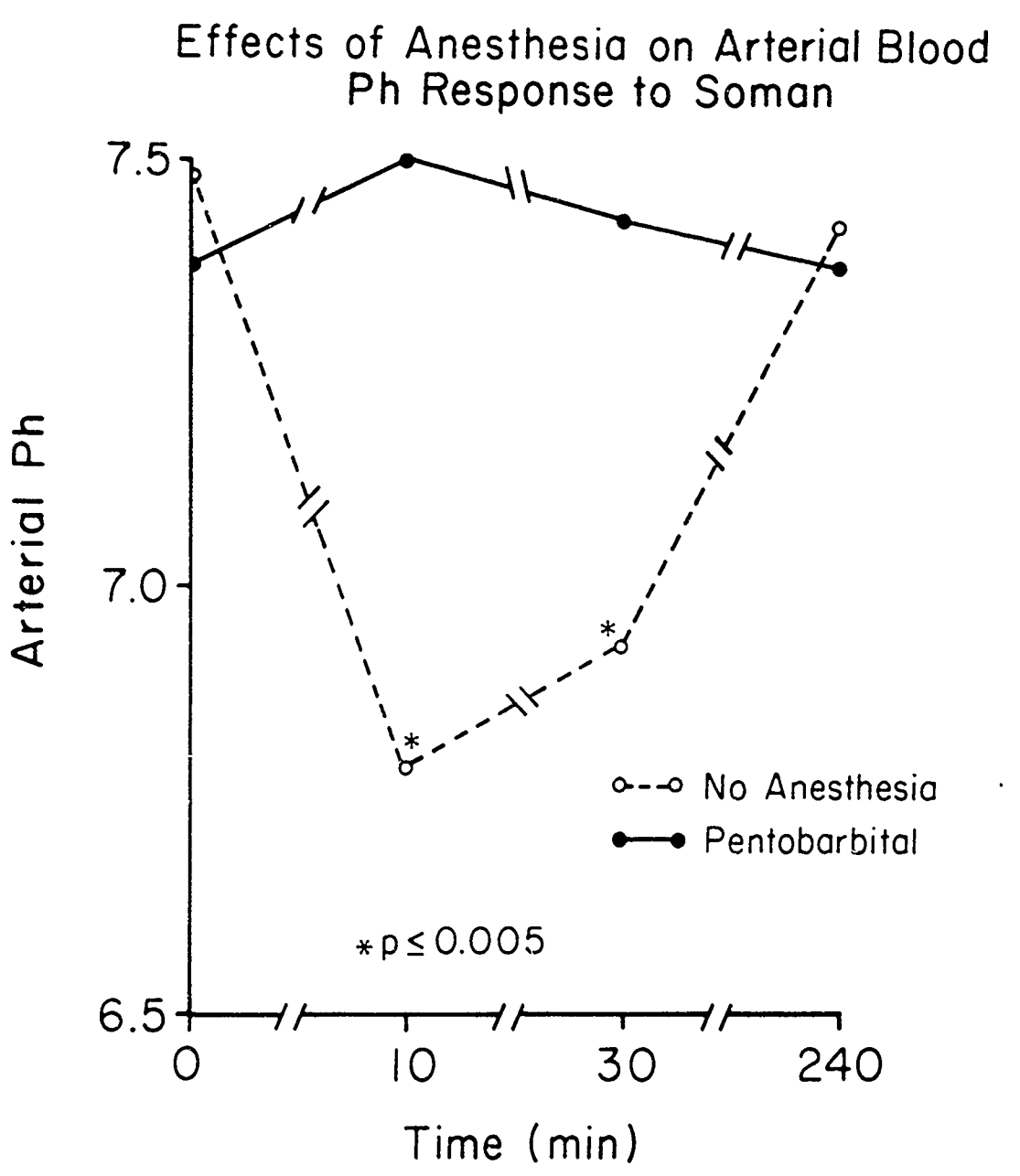
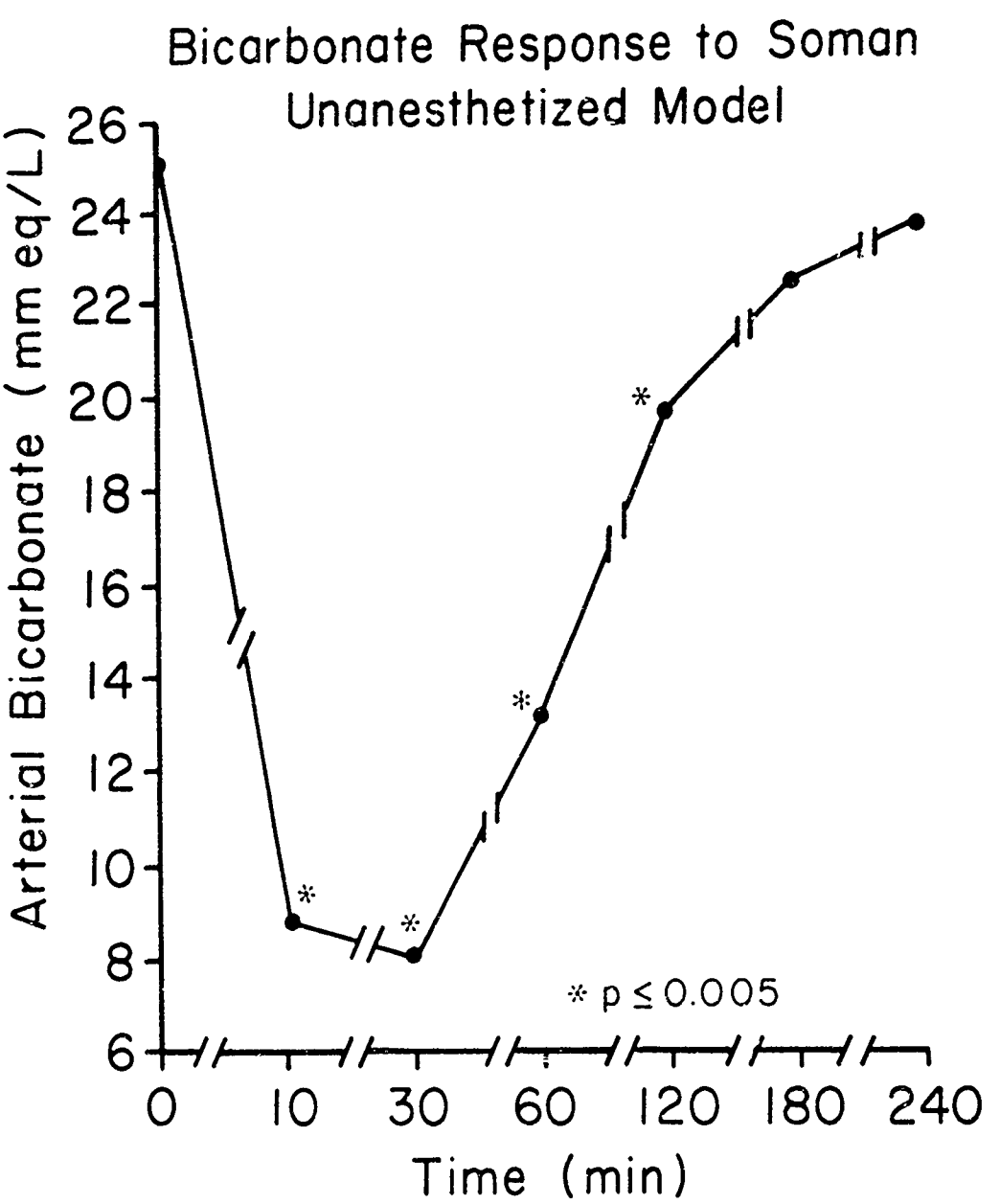


Blood Lactic Acid: Unanesthetized Animals

Baseline	0 mEq/L
Time of apnea (mean 7.9 min)	25.87 mEq/L (±4.47 S.E.M.)
1 hr post infusion	24.4 mEq/L (±8.52 S.E.M.)
4 hr post infusion	4.73 mEq/L (±2.74 S.E.M.)
Normal controls	0.5-2.2 mEq/L



Arterial bicarbonate (HCO_3) response to Soman in a group of six tethered, unanesthetized animals ventilated by HFJV, 100% O_2 via CTC. Soman (13.14 μg/kg) was infused over a 10 min period. A significant decrease in HCO_3 at 15 min coincided with an increase in serum lactic acid and lower arterial pH value.

SUMMARY

In Soman intoxicated baboons:

Pentobarbital

— Decreased muscle fasciculation
— Ameliorated lactic acidosis

but

— Potentiated cardiovascular effects

Pentobarbital did not alter:

— Secretions
— Onset of apnea
— Other respiratory effects

CONCLUSIONS

In the unanesthetized animal, Soman causes severe muscular fasciculation, metabolic acidosis, prolonged apnea and a mild decrease in blood pressure.

Pentobarbital anesthesia ameliorates the muscular activity and acidosis, but prolongs and potentiates the hemodynamic effects.

TOXICITY OF SOMAN--INHALATION EXPOSURE SYSTEM DESIGN

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INTRODUCTION

The primary objective of this research program is to define and quantify the sequence of pathophysiologic events associated with Soman intoxication. These events are being evaluated by three exposure routes: inhalation, subcutaneous infusion, and intravenous infusion. The evaluations are performed in three animal species: the dog, the monkey, and the cat. Each species is being evaluated by all three routes of administration.

Included in Phase I, the developmental phase, is the establishment of neat agent inhalation capabilities. These capabilities include a neat agent vapor generation system and an inhalation exposure system for each of the three species. The vapor generation system should provide stable exposure atmospheres for inhalation exposure of each species at exposure levels near the LCT_{50} for GD. The inhalation exposure system should provide the capability of exposing an unanesthetized subject of each species by nose- or mouth-only to vapors of GD in air. It should also maintain the animal safely restrained during, and after, the exposure period, and permit instrumentation of the test animal for various physiologic parameters. Finally, the design of the system should permit the accurate measurement of respiratory flowrate during inhalation challenge.

Both the vapor generation system and inhalation exposure system have been designed, developed, and tested. They have also been effectively utilized in inhalation exposures of 9 dogs, 9 cats, and 9 monkeys to GD vapors. Additionally, the exposure system has been utilized for restraint and respiratory flow measurements during the exposure of 33 cats, 45 dogs, and 36 monkeys by subcutaneous or intravenous infusion. This poster describes these two systems.

DESIGN CRITERIA

Our development of atmosphere generation and inhalation exposure systems addressed a number of critical factors. The following critical design criteria have been incorporated into the design of the two systems, and realized in their implementation:

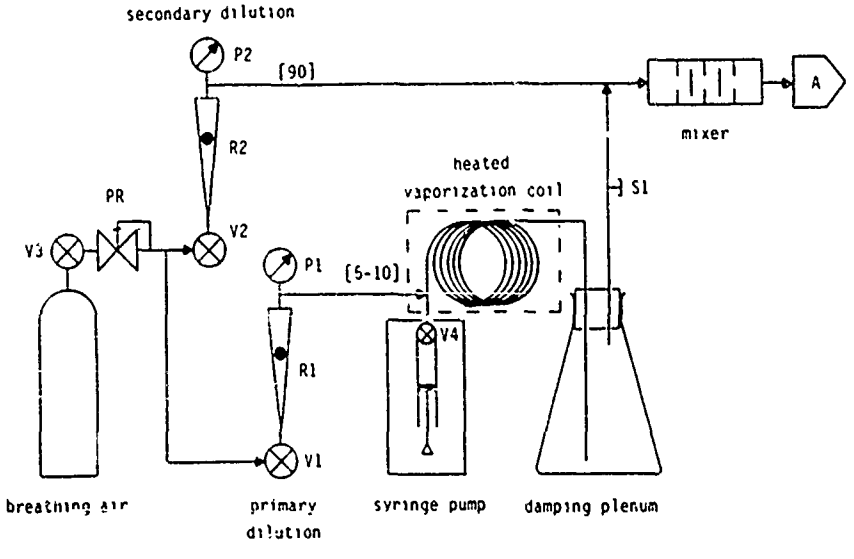
- Exposure duration should be ten minutes, commensurate with previous studies, and striking a balance between the accuracy of characterization of longer exposures and the minimization of stress to the test animal in shorter exposures.
- Available exposure concentrations should range from 10% of the lowest LCT_{50} to 200% of the highest for the three species of interest, circa 1 to 32 mg/m^3.
- Exposure concentration should be stable over the exposure period, to provide comparability between exposures.
- Actual exposure concentration should be measured during the exposure, and its variability determined.
- The exposure system should permit exposure by nose or mouth inhalation only, limiting the effects of skin absorbed GD and reducing the amount of exposed surface requiring decontamination.
- The test animal should be sufficiently restrained to permit safe unanesthetized exposure while permiting ease of attachment of physiologic monitoring equipment.
- The exposure system should permit the accurate measurement of respiratory flowrate without influencing it.
- The exposure system should provide the "fresh" test atmosphere to the animal at all times, so that interpretation of the results is not complicated by the mixing of inlet and exhaust gases.
- The systems should be safe to operate; utilization of GD should be efficient, restraint of test animals should be thorough, and containment of the test material should be complete.

VAPOR GENERATION SYSTEM

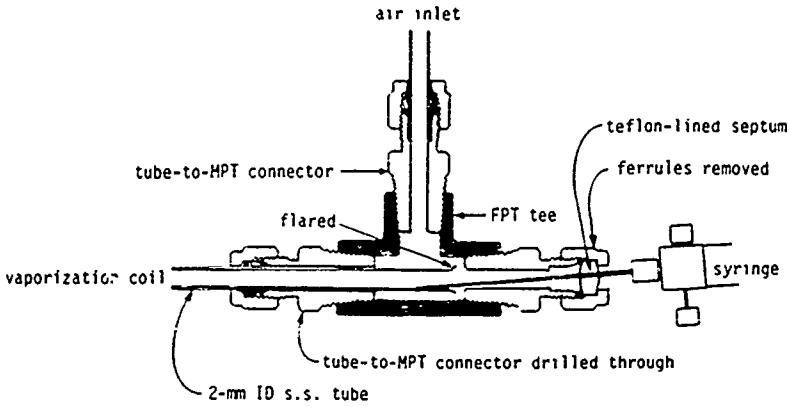
Neat GD is metered to the inlet of a vaporization coil of stainless steel tubing approximately 2 meters in length and 2 millimeters inside diameter. The feedrate is controlled precisely by a variable speed syringe pump fitted with a 100-uL gas-tight sampling syringe. This sampling syringe has a teflon-tipped plunger in a smooth glass barrel, which effectively prevents creep of volatile solvents; and a teflon and stainless steel valve, allowing the syringe to be sealed and safely handled after filling with neat agent.

The stainless steel syringe needle is inserted into the vaporization coil, through a teflon-lined septum, at an angle so that the tip touches the inner surface of the tube, maximizing the surface area for vaporization. Bottled breathing air is supplied to the inlet of the vaporization coil at a flowrate between 5 and 10 L/min. The coil is heated to a temperature which produces an air temperature at its exit of between 50 and 90 C at the operational air flowrates.

Downstream from the vaporization coil, the entrained GD vapor enters a glass damping plenum where continuous back-mixing reduces fluctuations in the generator output which would result from uneven syringe plunger movement or temporary droplet formation at the needle outlet. The vapor and air which exit the plenum are mixed with secondary dilution air at about 90 L/min, resulting in a total air flowrate from the vapor generation system of about 100 L/min. To ensure complete mixing of the entrained vapor with the secondary dilution air under laminar conditions, a mixer was inserted between the vapor generation and inhalation exposure systems. Following the mixer is a length of delivery tube, greater than six inside tube diameters in length, to allow the velocity profile to stabilize prior to entering the inhalation exposure system.



Vapor Generation System



Schematic Cross-Section of Vapor Entrainment Section

VAPOR GENERATION SYSTEM

Performance Evaluation

Trials with diethyl malonate were discontinued, in favor of GD trials, as soon as it was shown that analytical concentrations were comparable to nominal concentrations.

Early trials with neat GD showed uncertainty in the measured concentration of GD vapor produced during the first 20 minutes of generator operation. No simple modification of the generation system alleviated this concentration variability, though the measured concentration approached, and stabilized at, the nominal concentration by the end of the start-up period. The extremely low GD liquid feed rates, relatively large dead space in the entrainment section of the generator, and the slack in the syringe pump gear train probably account for this variability. By measuring GD concentration during the start-up period, we were able to withhold initiation of animal exposure until after concentration had stabilized.

The data presented are GD analytical concentration during trials on the last two trial dates before initiation of animal studies. They are typical of the temporal stability of the generator output.

Mean analytical concentrations were within 10% of the nominal concentrations for each period. Coefficients of variation (standard deviation/mean) for replicate analyses of standards analyzed on these dates ranged from 0.017 to 0.12. Coefficients of variation of analytic concentration for the four periods ranged from 0.0042 to 0.076, well within the analytic variability. No discontinuities in concentration were observed for the trial period.

Although the system has not been tested over its full range of design capabilities (1 to 32 ug/L) it has performed satisfactorily in trials from 5 to 20 ug/L and routinely in 27 actual inhalation exposures.

CHEMICAL ANALYSIS METHODOLOGY

Previous experience with GD at Battelle has shown gas chromatography (GC) with either flame photometric detection (FPD) or flame ionization detection (FID) to be sensitive and accurate techniques for quantification of GD vapor collected in impingers. In order to provide rapid response analyses, so that exposure concentration could be adjusted, we modified the analytic technique to allow gas-phase injection of samples collected with gas-tight sampling syringes directly to the GC injection port. Analysis time was decreased by modifying the standing methodologies for isothermal operation rather than temperature programming. GC-FPD was utilized for GD analyses, because of its enhanced sensitivity over GC-FID analysis.

Following are typical analytic conditions for GC-FPD analysis of GD vapors:

Gas chromatograph --
- Model: Varian, model 3700
- Detector: flame photometric detector (FPD)
- Filter: phosphorus

Temperatures --
- Injector: 220 C
- Column: 135 C, isothermal
- Detector: 220 C

Column --
- Column material: glass, 6 ft X 1/4 inch X 2 mm
- Packing: 3% QF-1 on 80/100 mesh Gas Chrom Q
- Standard solvent: 2-propanol
- Retention time: 1.1 min

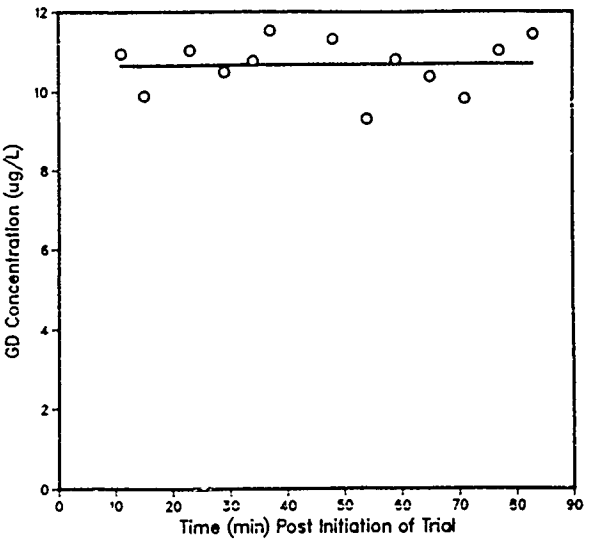
Gases --
- Carrier: He, 30 mL/min
- Flame: H_2, 140 mL/min
  Air_1, 80 mL/min
  Air_2, 170 mL/min

Under these conditions, analyses could be performed at intervals of less than three minutes with sample sizes of 300 uL. The peaks produced were well resolved and symmetrical.

During the development of the vapor generation system, diethyl malonate was used as a GD simulant. Since its physical characteristics are similar to GD, diethyl malonate was also conveniently measured by GC-FID analysis of gas-phase samples. The major differences from the GD methodology are a column of 20% SP2100/0.1% CW1500 on 100/120 mesh Supelcoport, and a retention time of 3.1 minutes.

Summary of GD concentrations for trial on 4/20/84.

Time Post t_0 (min)	GD Concentration (ug/L)
11	11.0
15	9.9
23	11.0
29	10.5
34	10.8
37	11.5
48	11.3
54	9.3
59	10.8
65	10.4
71	9.8
77	11.0
83	11.4
Mean	10.7
sd[1]	0.70
CV[2]	0.066



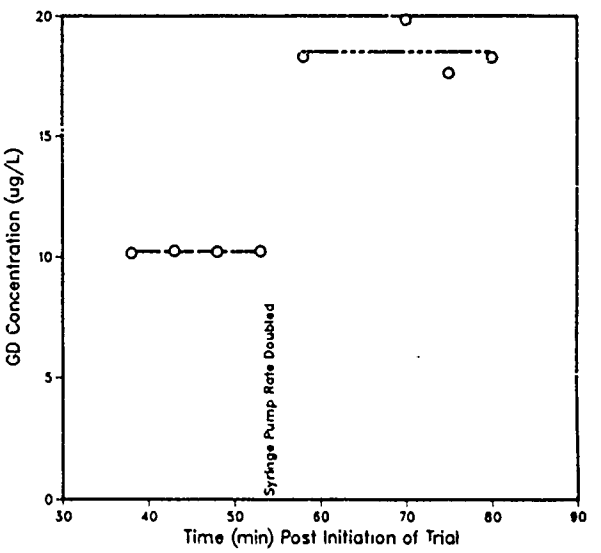
Summary of GD concentrations for trial on 4/24/84.

Period 1 - Initial Conditions

Time Post t_0 (min)	GD Concentration (ug/L)
38	10.2
43	10.3
48	10.2
53	10.2
Mean	10.2
sd[1]	0.04
CV[2]	0.004

Period 2 - Syringe Pump Rate Doubled

Time Post t_0 (min)	GD Concentration (ug/L)
58	18.3
70	19.8
75	17.6
80	18.3
Mean	18.5
sd	0.95
CV	0.051



[1]Standard deviation.
[2]Coefficient of variation.

INHALATION EXPOSURE SYSTEM

Delivery System

The test atmosphere is supplied to the exposure system at a nominal flowrate of about 100 L/min. It is drawn through the exposure mask at a constant bias flowrate of 30 L/min. This bias flow provides a continuous purge of the exposure mask with the fresh test atmosphere. The remaining 70 L/min of overflow is released to the hood exhaust. The overflow ensures a sufficient supply of the test atmosphere during peak inspiratory flowrates.

A septum (S2) in the supply line gives access for sampling of the test atmosphere prior to initiation of exposure, for concentration measurement and adjustment.

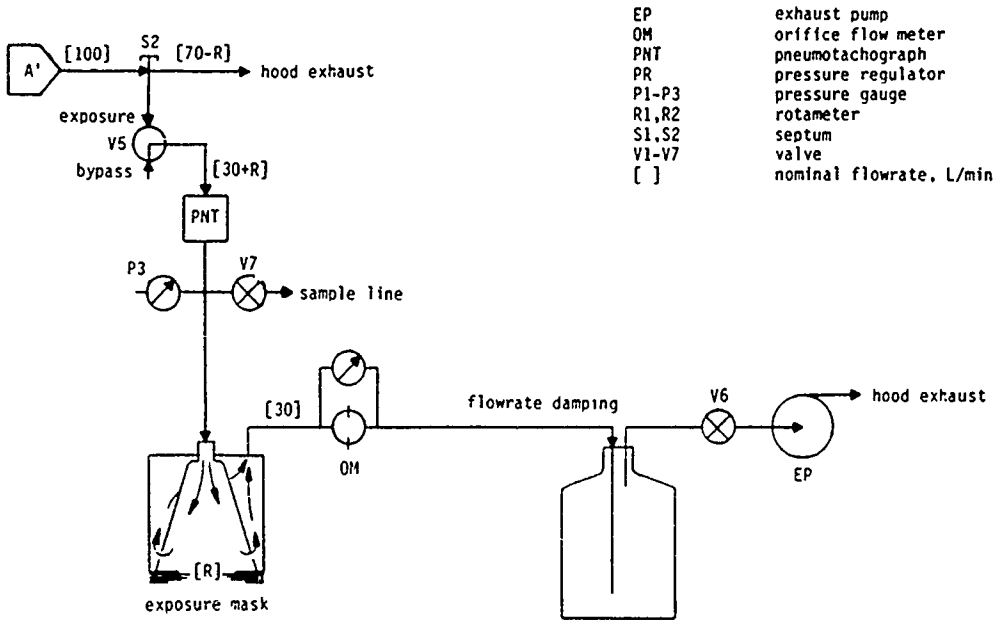
A three-way valve (V5) switches the inlet of the exposure mask between the test atmosphere and clean room air. The common port of V5 connects to the inlet of a pneumotachograph (PNT), the outlet of which connects to the inlet of the exposure mask. A pressure tap in the line between the PNT and the exposure mask provides access for measurement of mask pressure. The PNT measures total mask flowrate, so that respiratory flowrate can be calculated as the difference between total mask flowrate and bias flowrate.

The exposure mask was designed to provide exposure by nose and mouth inhalation while providing suitable restraint of the test animal and allowing measurement of respiratory flowrates and mask pressure. The mask is constructed of an anesthesia cone enclosed in an exhaust plenum. The exposure atmosphere is drawn into the inlet of the cone, past the nose and mouth of the test animal, and through a series of perforations at the base of the cone into the exhaust plenum before being drawn out the exhaust port. This arrangement causes inspired air to be drawn from the stream of fresh test atmosphere at the inlet of the mask and exhaled air to be swept to the exhaust perforations at the base of the mask.

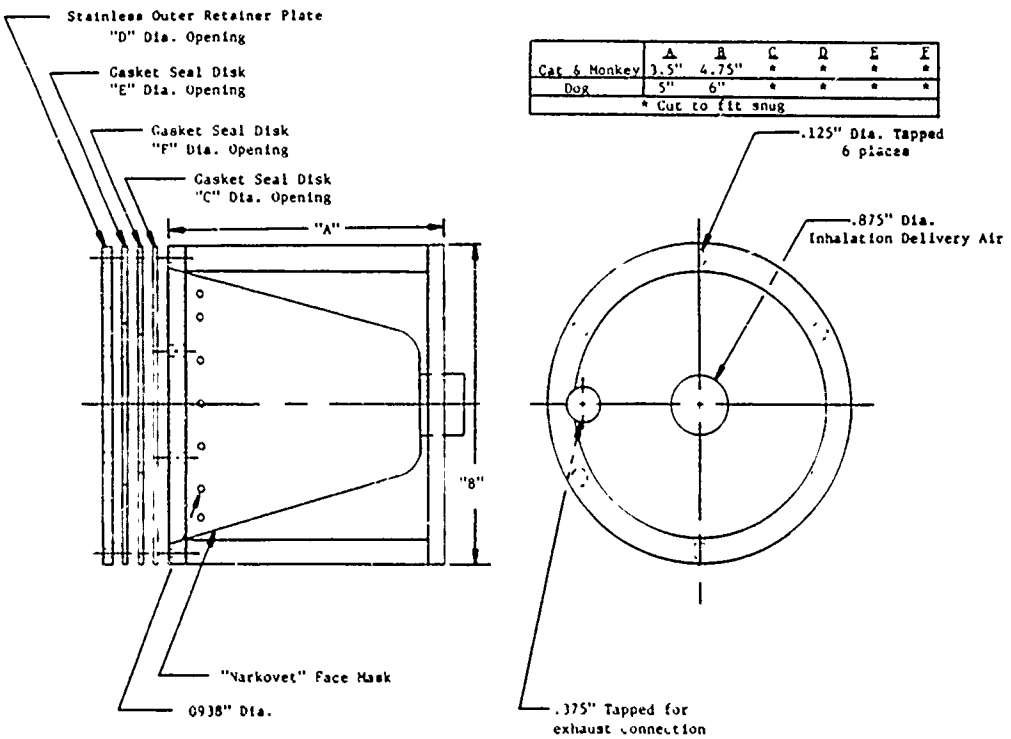
The nose port of the mask is constructed of a stainless steel plate with a centrally located circular hole, 3 3/4 inches diameter for dogs or 3 1/4 inches diameter for cats and monkeys. An air-tight seal between the base of the animals snout and the nose port was obtained by backing the port with a series of separate thin latex rubber gaskets, each with a centrally located opening. The openings are approximately circular for cats and monkeys, and eliptical for dogs, and are individually cut to provide a very snug fit for the test animal.

The air exhausted from the mask is drawn through a thin-plate orifice flowmeter and a flow control valve by a diaphragm pump. The flowmeter and valve provide a means to set the bias flowrate. Pulsations in the bias flowrate caused by the diaphragm pump were effectively damped by inserting a 4-liter plenum between the valve and flowmeter.

All transport lines between the mixer and the exposure mask are at least 3/4 inch inside diameter, to minimize system pressures which might effect the respiratory response of the test animal. The exhaust from the diaphragm pump is delivered to the hood exhaust. Drawing the test atmosphere with an exhaust pump maintains the delivery system at a slight negative pressure relative to ambient conditions, preventing any accidental release of GD.



Inhalation Exposure System



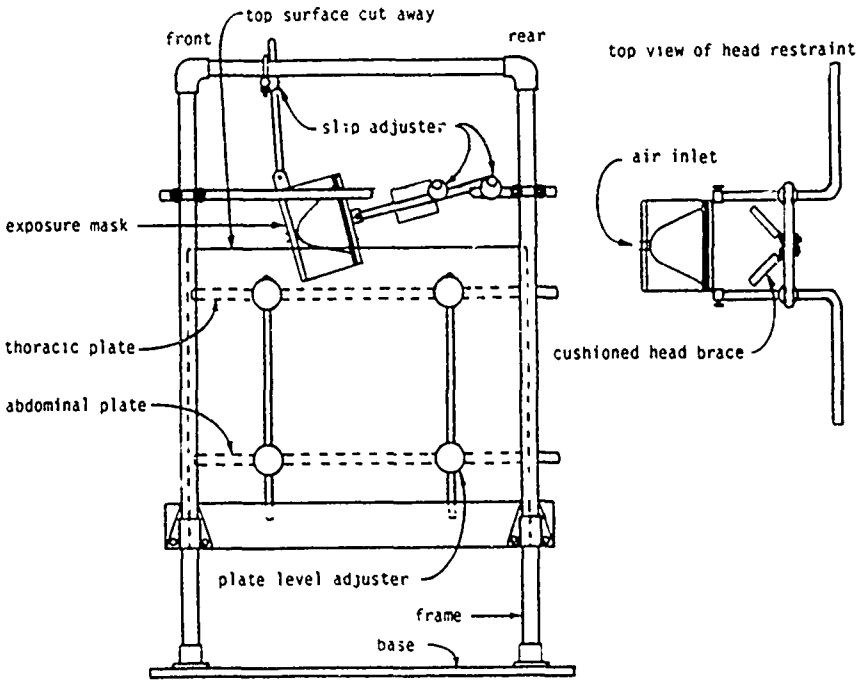
Nose-Only Exposure Mask

Animal Restraint System

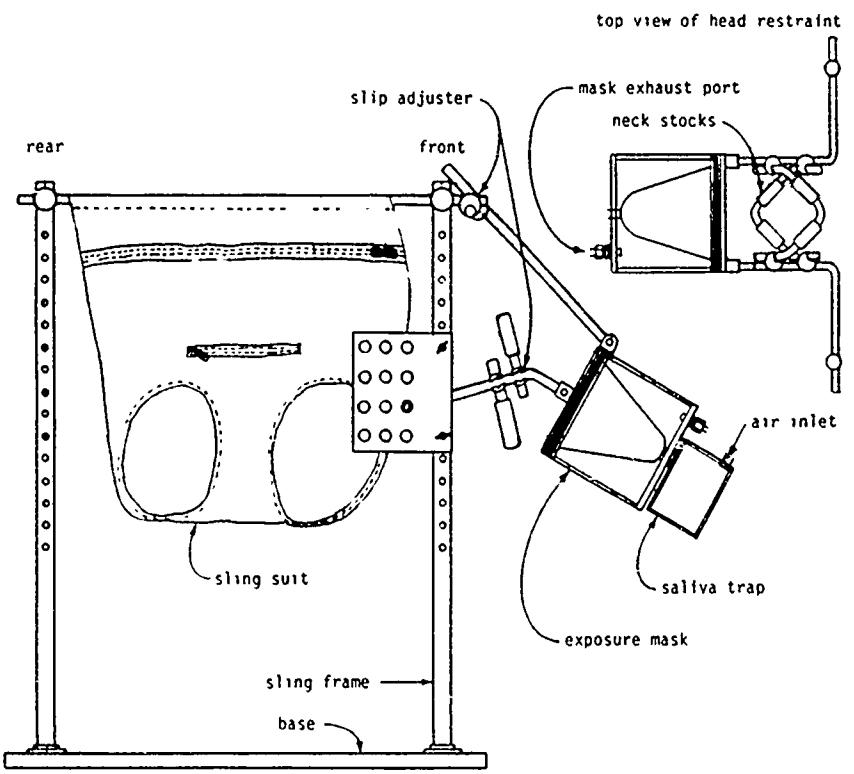
Cats and dogs were effectively restrained with a system consisting of three major components: a commercial sling suit to keep physiologic transducers in place while providing a means for attachment of the animal to the rest of the restraint system, a Battelle-constructed sling frame to elevate the animal above the work surface and provide support for the head restraint, and a head restraint to maintain the head correctly interfaced to the exposure mask.

Neck stocks are attached firmly to the exposure mask, and configured to produce firm pressure behind the occipital bone and slight pressure under the parietal bone. Slip adjusters allow the restraint to be adjusted to individual animals so that the nose is accurately positioned within the exposure mask and cannot be withdrawn. This arrangement allows the animal to breath freely by either nose or mouth while maintaining a complete seal at the base of the snout.

Monkeys were restrained in a commercial primate restraint chair modified to accept a head restraint and an exposure mask. The neck stocks were removed and the top surface cut down to provide access for the exposure mask. A thoracic plate prevents the monkey from pulling down below the plane of the mask, while the mask and an angle brace behind the head provide the necessary lateral head restraint. The arrangement is fully adjustable for various head and body sizes, as well as degree of head tilt. Adjustment of the head brace can be made to provide accurate positioning of the exposure mask over the snout of the animal so that the test animal can breath freely by either nose or mouth while a complete seal is maintained under the most violent struggling.



Monkey Exposure Restraint Device



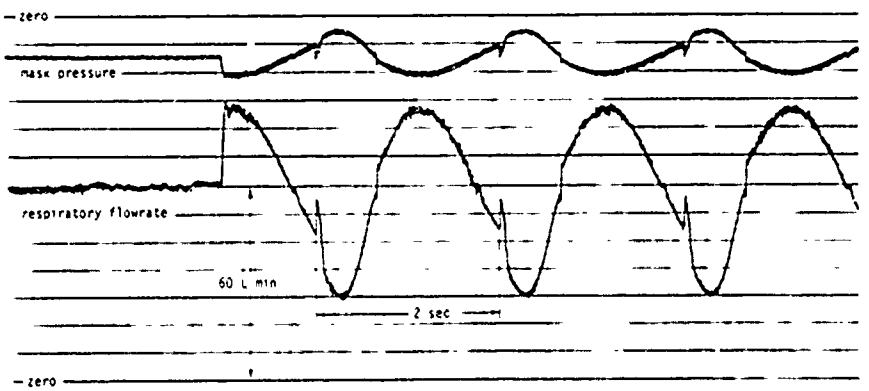
Cat/Dog Exposure Restraint Device

INHALATION EXPOSURE SYSTEM

Performance Evaluation

The restraints and exposure masks have been used in actual inhalation exposures of 9 cats, 9 dogs, and 9 monkeys. They have also been used to provide restraint and respiratory flow measurement during the exposure of 33 cats, 45 dogs, and 36 monkeys by subcutaneous or intravenous infusion. In all cases, test animals remained safely under control for the duration of the exposure period.

To evaluate the ability to monitor mask pressure and respiratory flowrate in the presence of the bias flow, and to assess the effectiveness of pump pulsation damping, a respirator pump was interfaced to the nose port of the mask, simulating test animal respiration. Exhaust pump output was increased to twice the design flowrate to exaggerate its impact. Mask pressure and mask inlet flowrate were recorded under various respirator pump duty cycles and examined for pump pulsations, baseline drift, and noise. As shown, pump pulsations, drift, and noise were insignificant, relative to respiratory flowrate. This was true under all respirator pump operating conditions.



Mask Pressure and Inlet Flowrate
During Respiratory Simulation

SUMMARY

Pursuant to our requirements for neat agent inhalation capabilities in our study of the toxicity of Soman, we have designed, developed, and tested systems for both vapor generation and inhalation exposure. The vapor generation system provides stable exposure atmospheres for inhalation exposure of cats, dogs, or monkeys at exposure levels near the LCT_{50} for GD. The inhalation exposure system provides the capability of exposing an unanesthetized subject of any of the species by nose- and mouth-only to vapors of GD in air. The test animal is maintained safely restrained during and after the exposure period. The animal can be conveniently instrumented for various physiologic parameters, while the exposure system provides a means for the accurate measurement of respiratory flowrates concurrent with exposure.

Vapor generation is accomplished by pumping neat GD, with a syringe pump, at a known and constant rate onto the mildly heated (circa 50 C) inner surface of a vaporization coil in a stream of air at a constant flowrate.

Dogs and cats are restrained in a commercial sling suit and sling frame fitted with a head restraint consisting of neck stocks and an inhalation exposure mask. Monkeys are restrained in a commercial primate restraint chair fitted with braces at the rear of the head and a similar exposure mask.

The inhalation exposure mask provides exposure of the nose and mouth to a continuous stream of the "fresh" test atmosphere at a constant flowrate. This material enters the front end of the mask, passes around all sides of the snout of the test animal, and is exhausted through a series of perforations at the base of the snout. Respiratory flowrate is conveniently and accurately measured as the difference between the inlet flowrate and the constant exhaust flowrate.

Validation studies have shown the vapor generation system to rapidly achieve stable exposure concentrations. Although not tested over the complete range of its design capabilities (1 to 32 ug/L) it has performed satisfactorily in trials at concentrations from 5 to 20 ug/L. The vapor generation system and exposure system have been successfully utilized in actual inhalation exposures of 9 dogs, 9 cats, and 9 monkeys to vapors of GD. The exposure system has also been used to provide restraint and respiratory flow measurement during the exposure of 33 cats, 45 dogs, and 36 monkeys by subcutaneous or intravenous infusion. In all cases, test animals remained safely under control for the duration of the exposure period, while respiratory flowrate was effectively measured.

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THE PATHOPHYSIOLOGY OF ACUTE SOMAN INTOXICATION IN THE CYNOMOLGUS MONKEY VIA THREE ROUTES OF EXPOSURE

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OBJECTIVES

THIS RESEARCH IS DESIGNED TO DEFINE AND QUANTIFY THE SEQUENCE OF PATHOPHYSIOLOGIC EVENTS ASSOCIATED WITH SOMAN INTOXICATION. THESE EVENTS ARE BEING STUDIED IN THREE SPECIES (DOG, MONKEY AND CAT) BY THREE ROUTES OF ADMINISTRATION; INTRAVENOUS, SUBCUTANEOUS AND INHALATION. THE DEFINITION OF THE SEQUELAE OF SOMAN INTOXICATION IS ACCOMPLISHED BY CONTINUOUS MEASUREMENT AND THE SUBSEQUENT ANALYSIS OF RESPIRATORY, CARDIOVASCULAR AND NEUROMUSCULAR FUNCTION. THE IMPACT OF CHEMICAL RESTRAINT IS ELIMINATED BECAUSE THE ANIMAL MODELS USED IN THIS STUDY ARE NOT ANESTHETIZED DURING THE EXPOSURE TO SOMAN. IDENTICAL PHYSIOLOGIC MEASUREMENTS AND ADMINISTRATION PROTOCOLS ARE UTILIZED IN ALL THREE SPECIES SO THAT A COMPARATIVE ANALYSIS CAN BE MADE NOT ONLY AMONG ROUTES OF EXPOSURE BUT AMONG SPECIES. THIS PARTICULAR POSTER DESCRIBES ONLY THE EVALUATION OF CYNOMOLGUS MONKEY DATA BY THREE ROUTES OF EXPOSURE.

METHODS

TWENTY-SEVEN CYNOMOLGUS MONKEYS WERE EVALUATED DURING A 10 MINUTE CHALLENGE AT A NEAR-LETHAL CONCENTRATION OF SOMAN FOLLOWED BY A MAXIMUM OF A 50 MINUTE PHYSIOLOGIC MONITORING PERIOD. THE ANIMALS WERE DISTRIBUTED AMOUNG THREE ROUTES OF EXPOSURE. THE INTRAVENOUS AND SUBCUTANEOUS ROUTES WERE 10 MINUTE, SLOW INFUSIONS OF DILUTE AGENT; WHEREAS THE INHALATION CHALLENGE INVOLVED A 10 MINUTE EXPOSURE TO NEAT AGENT.

STANDARD ASEPTIC SURGICAL TECHNIQUES WERE EMPLOYED TO IMPLANT THE VARIOUS TRANSDUCERS. CANNULAE WERE IMPLANTED AND EXTENDED TRANSCUTANEOUSLY. FOR TRACHEAL PRESSURE, A CATHETER WAS PLACED IN THE TRACHEA. INTRAPLEURAL PRESSURE WAS MEASURED WITH A BALLOON IN THE PLEURAL SPACE. ARTERIAL BLOOD SAMPLES AND PRESSURES WERE OBTAINED BY AN ARTERIAL CATHETER PLACED IN THE AORTA. AN ELECTROMAGNETIC FLOW METER WAS PLACED ON THE ASCENDING AORTA. NEUROMUSCULAR AND ELECTRO-PHYSIOLOGIC PARAMETERS WERE EVALUATED BY IMPLANTING: A DIA-PHRAGMATIC ELECTRODE, A CORTICAL EEG ELECTRODE, AN EPICARDIAL SURFACE ECG ELECTRODE, AND A CUFF-TYPE PHRENIC NERVE ELECTRODE. RESPIRATORY FLOWS WERE MADE UTILIZING A MASK WHICH SURROUNDED THE ANIMAL'S NOSE AND MOUTH. A CONTINUOUS ONE-WAY FLOW OF 30 LITERS PER MINUTE PROVIDED BIAS FLOW TO ELIMINATE DEAD SPACE. RESPIRATORY FLOWS WERE CALCULATED BY THE DIFFERENCE BETWEEN INLET AND BIAS FLOWS. THIS MASK SYSTEM ALSO PROVIDED AN EXPOSURE ROUTE DURING INHALATION PROCEDURES. ANIMALS WERE PLACED IN A SLING-TYPE RESTRAINING DEVICE. TRANSDUCERS AND LEAD WIRES WERE ATTACHED TO AN ELECTRONICS FOR MEDICINE VR12 PHYSIOLOGIC DATA RECORDER AND A SANGAMO SABER 6 FM TAPE RECORDER. DATA WAS DIGITIZED UTILIZING A PDP-1144 COMPUTER.

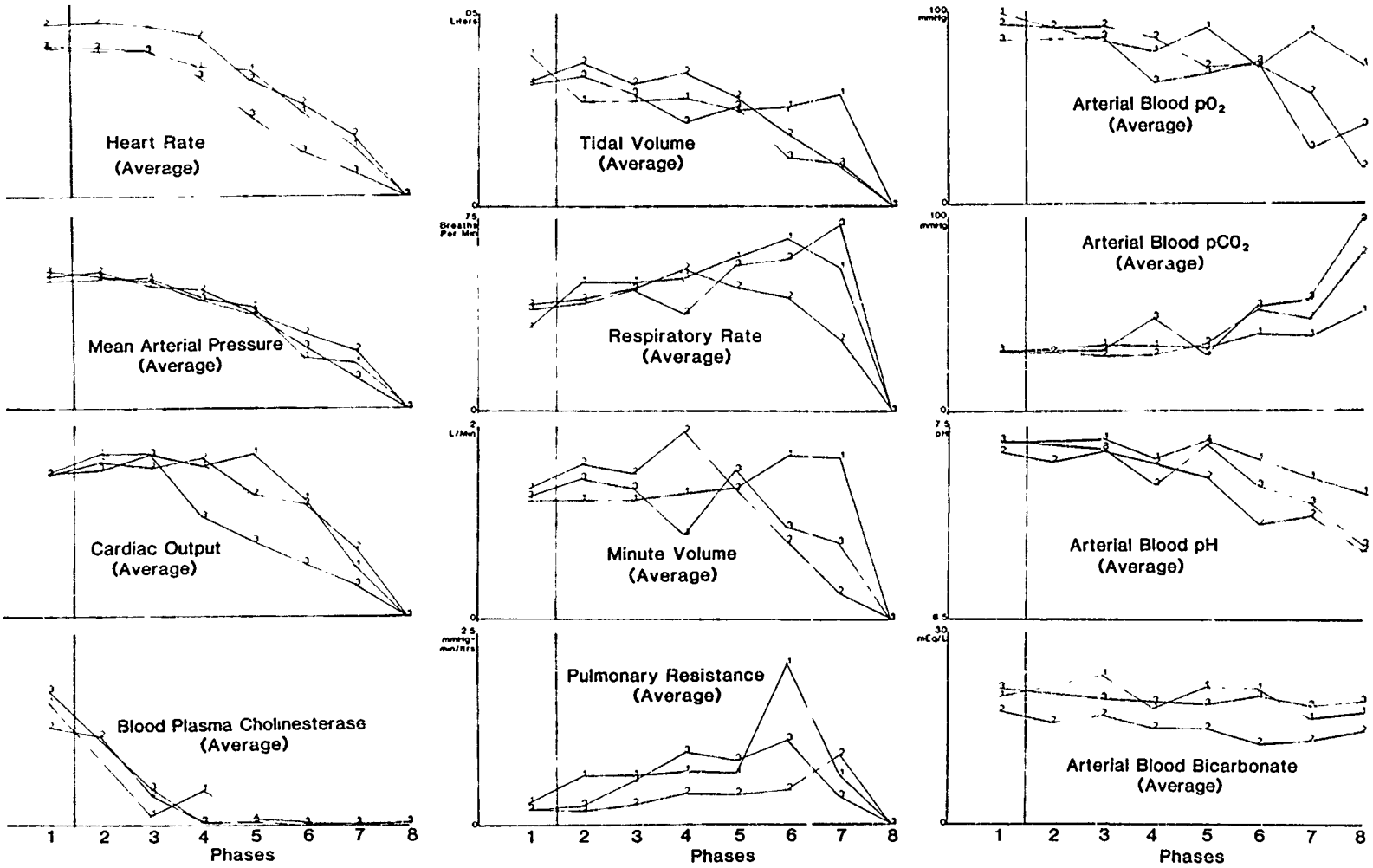
ALL DIGITAL DATA WERE NORMALIZED IN TIME BY DIVIDING THE EXPERIMENT INTO 8 PHASES. THE FIRST PHASE WAS BASELINE AND THE LAST PHASE DEATH. THE SIXTH PHASE WAS IDENTIFIED AS THE BEGINNING OF THE RAPID DECLINE IN AORTIC PRESSURE. EACH POINT REPRESENTS THE AVERAGE RESPONSE FOR THE INDICATED VARIABLE WITHIN THAT PHASE FOR ALL ANIMALS WITHIN A PARTICULAR EXPOSURE GROUP. THIS METHOD OF DATA COLLECTION ACCOUNTS FOR DIFFERENCES IN EXPERIMENT LENGTH.

HORIZONTAL AXIS
TIME ADJUSTED EXPERIMENTAL PHASES

Phase 1=Baseline
Phase 3=Early Response
Phase 5=Middle Response
Phase 7=Late Response
Phase 8=Death

VERTICAL AXIS
AVERAGE RESPONSES

Variable 1=Inhalation Monkeys (N=5)
Variable 2=Subcutaneous Monkeys (N=6)
Variable 3=Intravenous Monkeys (N=8)



PRELIMINARY EVALUATION OF ALL THREE TIME ADJUSTED AVERAGE TRACINGS AND ALL VARIABLES INDICATE A GREAT SIMILARITY AMONG ALL THE ROUTES OF EXPOSURE. INHALATION EXPOSURE MEAN TIME TO DEATH WAS 19.8 ± 7 MINUTES. SUBCUTANEOUS INFUSION MEAN TIME TO DEATH WAS 33.4 ± 12 MINUTES. THE INTRAVENOUS INFUSION MEAN TIME TO DEATH WAS 22.8 ± 6.6 MINUTES.

IN THE EARLY PHASES, MEAN SYSTEMIC ARTERIAL PRESSURE AND HEART RATE DECREASED SLIGHTLY WHILE CARDIAC OUTPUT REMAINED RELATIVELY CONSISTENT. THUS, SYSTEMIC VASCULAR RESISTANCE DECREASED DURING THIS PERIOD. OCCASIONAL VENTRICULAR AND SUPERVENTRICULAR PREMATURE BEATS OCCURRED IN ALL ROUTES OF EXPOSURE. ALTHOUGH TIDAL VOLUME DECREASED VERY SLIGHTLY, RESPIRATORY FREQUENCY INCREASED SLIGHTLY SO THAT MINUTE VENTILATION REMAINED UNCHANGED. AIRWAYS RESISTANCE INCREASED SLIGHTLY VIA INHALATION AND INTRAVENOUS EXPOSURE.

IN THE MIDDLE PHASE, MEAN SYSTEMIC ARTERIAL PRESSURE AND HEART RATE CONTINUED TO DECREASE. CARDIAC OUTPUT WAS SUSTAINED NEAR CONTROL VALUES IN THE INHALATION ANIMALS BUT DECREASED IN THE SUBCUTANEOUS AND INTRAVENOUS ANIMALS. MINUTE VENTILATION AND ARTERIAL pCO_2 WERE SUSTAINED AT NEAR CONTROL LEVELS. RESPIRATORY FREQUENCY REMAINED SOMEWHAT ELEVATED WHILE TIDAL VOLUME AND ARTERIAL pO_2 REMAINED SOMEWHAT BELOW NORMAL. AIRWAYS RESISTANCE WAS SLIGHTLY ELEVATED.

IN THE LATE PHASE, MEAN SYSTEMIC ARTERIAL PRESSURE, CARDIAC OUTPUT, AND HEART RATE DECREASED PRECIPITOUSLY. ATTENDING THE PROFOUND BRADIACARDIA WAS SECOND DEGREE A-V HEART BLOCK PUNCTUATED BY SLOW JUNCTIONAL RHYTHM AND FINALLY A SLOW IDIOVENTRICULAR RHYTHM AND DEATH. MINUTE VENTILATION AND pO_2 DECREASED WHILE RESPIRATORY FREQUENCY AND TIDAL VOLUME DECREASED REMARKEDLY. pCO_2 AND AIRWAYS RESISTANCE INCREASED. IN THE LATE PHASE, ARTERIAL pO_2 APPEARED TO BE BETTER SUSTAINED IN THE INHALATION ANIMALS. THIS WAS SUBSTANTIATED BY A HIGHER RELATIVE MINUTE VOLUME PROVIDED BY A HIGHER TIDAL VOLUME. HOWEVER, THERE WAS ALSO A HIGH AIRWAYS RESISTANCE IN THESE ANIMALS.

DISCUSSION

SEVERAL HYPOTHESES CAN BE GENERATED AS TO THE MECHANISM UNDERLYING THE ABOVE OBSERVATIONS.

IN THE EARLY PHASES, CHANGES COULD BE ATTRIBUTED TO MECHANISMS OF PERIPHERAL STIMULATION DIRECTLY BY SOMAN OR THE BUILDUP OF ACETYLCHOLINE CAUSING SYSTEMIC ARTERIOLES TO DILATE, DECREASING SYSTEMIC PRESSURE WITH NO CHANGE IN CARDIAC OUTPUT. THE ARRHYTHMIAS COULD BE EITHER A CENTRAL OR A LOCAL MECHANISM. THE INCREASED FREQUENCY OF VENTILATION MAY HAVE BEEN A RESPONSE TO BRONCHO-CONSTRICTION (AS INDICATED BY AN INCREASE IN AIRWAYS RESISTANCE) AND A SLIGHT DECREASE IN COMPLIANCE. SINCE THERE ARE ONLY SLIGHT BLOOD GAS DERANGEMENTS, THE MILD TACHYPNEA COULD NOT BE DUE TO ALTERED BLOOD GASES. THE SLIGHT FALL IN PaO_2 WITH NO CHANGE OF VENTILATION OR $PaCO_2$ COULD RESULT FROM A SLIGHT $\dot{V}/\dot{Q}$ ABNORMALITY, SIMILAR TO THAT OBSERVED IN THE DOGS EXPOSED TO SOMAN.

THE CHANGES IN THE MIDDLE PHASE MAY BE EXPLAINED BY INCREASING VAGAL EFFERENT TRAFFIC OR DIRECT ACTION OF ACETYLCHOLINE OR SOMAN ON EFFECTOR ORGANS (I.E. SA NODE, SMOOTH MUSCLE OF BLOOD VESSELS OR AIRWAYS). $PaCO_2$ REMAINED RELATIVELY UNCHANGED THUS INDICATING RELATIVELY NORMAL ALVEOLAR VENTILATION DESPITE TIDAL VOLUME REDUCTION.

IN THE LATE PHASE NEAR SIMULTANEOUS AND PRECIPITOUS DEPRESSION OF BOTH CARDIOVASCULAR AND VENTILATORY PARAMETERS ARE BEST EXPLAINED AS A CENTRAL ACTIVITY UPON THE RESPECTIVE CENTERS. THIS PRESUMPTION IS MADE SINCE AIRWAYS RESISTANCE DID NOT ELEVATE SUFFICIENTLY TO LOAD BREATHING. FURTHER, ANALYSIS OF THE AORTIC PRESSURE CURVES INDICATE DIASTOLIC PRESSURE FELL DRAMATICALLY DUE TO BOTH BRADIACARDIA AND THE LOW SYSTEMIC VASCULAR RESISTANCE. HOWEVER, THE RATE OF RISE OF THE PRESSURE PULSE REMAINED GREAT INDICATING A RELATIVELY STIFF AORTA AND A COMPETENT VENTRICLE. FURTHER, PHRENIC AND EMG ACTIVITY CEASE JUST PRIOR TO CARDIAC STANDSTILL.

THUS:

EARLY

- CARDIAC IRRITABILITY
- DECREASED VASCULAR RESISTANCE
- SMALL $\dot{V}/\dot{Q}$ ABNORMALITY

MID

- DEPRESSED CARDIAC FUNCTION
- NO CARDIAC IRRITABILITY
- MINUTE VENTILATION NORMAL
- SLIGHT HYPOXIA
- SUSTAINED $\dot{V}/\dot{Q}$ ABNORMALITY

LATE

- SEVERE DEPRESSION OF CARDIO RESPIRATORY FUNCTION (CENTRAL IN ORIGIN?)

CENTRALLY-MEDIATED INCREASES IN BLOOD PRESSURE FOLLOWING ORGANOPHOSPHATE AGENTS

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ABSTRACT

Intravenous injection of soman (SO), sarin (SA), and DFP caused immediate centrally-mediated increases in blood pressure in urethane-anesthetized rats. Threshold doses for SO, SA and DFP were 10, 16 and 350 ug/kg, while maximum pressor responses followed doses of about 40, 50 and 2000 ug/kg, respectively. Blood pressure peaked at 40-50 mmHg within 30 sec after SO and SA. The rise was more gradual following injection of DFP, acheiving a maximum increase of almost 70 mmHg after 4 to 5 minutes. The maximum response was limited by death, which occurred at lower pressure rises with SO and SA than with DFP. Increases in blood pressure also followed s.c., i.m., and i.p. injection of soman, but were slower in onset and required higher doses. The magnitude of the pressor response to i.v. injected soman was closely correlated with the degree of cholinesterase inhibition in the cortex, hypothalamus and brainstem, but not in the striatum or plasma.

The relative inhibitory concentrations for SO, SA, and DFP on eel AChE were 1, 3 and 7000. CCl_3/H_2O partition coefficients, determined by measuring the fraction of their AChE inhibiting capacity remaining in the aqueous phase, were 150, 25 and 60. The K_2 values were 8 and 2 ($x10^7$) for SO and SA, respectively, and $1x10^4$ for DFP.

The pressor response was aborted or prevented by atropine (2 mg/kg) or phenoxybenzamine (10 mg/kg), but not by methylatropine. Atropine, but not phenoxybenzamine, increased acute survival following an LD_{50} dose of soman. The response also was prevented or aborted by i.v. administration of the selective central muscarinic antagonist DKJ-21 [N-(4-diethylamino-2-butynyl)-succinimide], at a dose of 40 mg/kg. This dose of DKJ-21 did not block the hypotensive response to i.v. injection of acetylcholine.

Sarin (15 ug) increased blood pressure by about 40 mmHg following injection into the cerebral ventricles. Prior intraventricular injection of pralidoxime (150 ug) prevented the pressor response.

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INTRODUCTION

Administration of various centrally active inhibitors of cholinesterase results in a rise in arterial pressure. This response results from the stimulation by acetylcholine of muscarinic receptors in the brain, which leads to an increase in sympathetic nerve activity. Recent work in our laboratory has demonstrated a close correlation between the magnitude of the pressor response and the degree of cholinesterase inhibition in specific brain regions.

The purpose of these experiments was to compare the cardiovascular responses to the cholinesterase inhibitors Soman, Sarin, and DFP.

METHODS

Male Sprague-Dawley rats weighing 250-310 g were anesthetized with urethane (1.3 g/kg, i.p.). A PE-50 cannula, prefilled with heparin (100 units/ml saline), was inserted into the right common carotid artery to record blood pressure. A second cannula was inserted into the left jugular vein to facilitate i.v. injection of drugs.

Blood pressure was recorded on a Watanabe recorder by means of a Statham pressure transducer coupled to the carotid cannula. Mean arterial pressure (MAP) was calculated as:

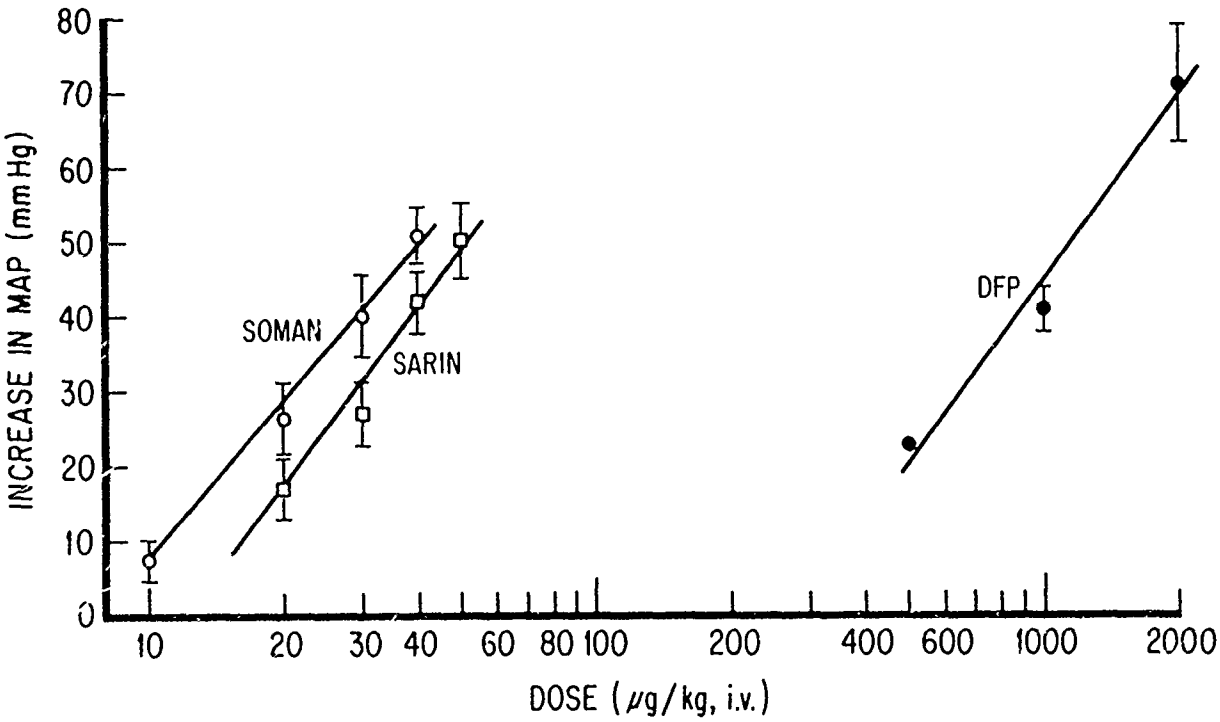
MAP = diastolic pressure + 1/3 pulse pressure.

Heart rate was monitored via a cardiotachometer triggered by the pressure pulse.

For intracerebroventricular (icv) injection of drugs, the rat was placed in a stereotaxic instrument (DKI) and a burr hole was drilled 1.5 mm lateral to bregma. An injection cannula (30 gauge hypodermic tubing) was lowered to a depth 3.5 mm below the cortex.

Acetylcholinesterase activity was determined using the spectrophotometric method of Ellman et al. Brain tissue was homogenized in 100 mM phosphate buffer (pH=8.0) at a concentration of 100 mg/ml. An 8 ul aliquot of the homogenate was added to a microcuvette containing 1.02 ml of 100 mM phosphate buffer (pH=8.0) and 40 ul of 10 mM dithiobisnitrobenzoic acid (DTNB). An 8 ul volume of acetylthiocholine (75 mM) then was added, the solution mixed, and the change in absorbance was measured at 412 u.

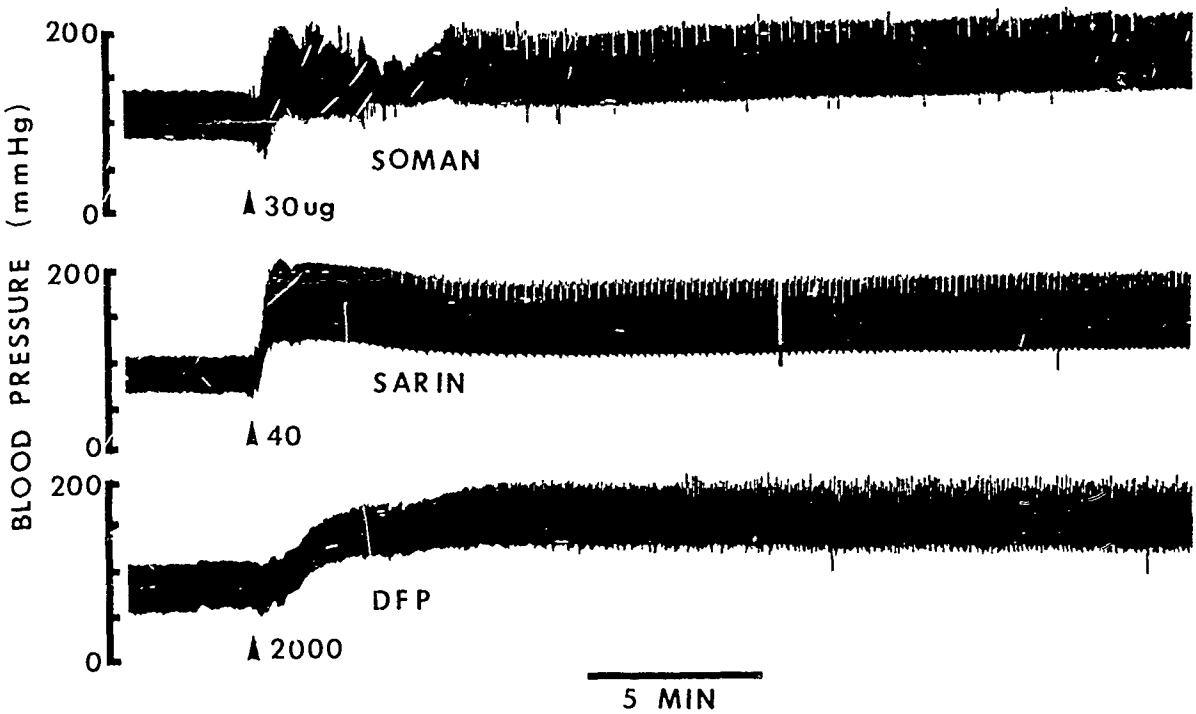
All drugs were dissolved in 0.9% saline. For injections into the lateral cerebral ventricle the volume of injection was 10 ul. For systemic injections, the volume of injection was 1 ml/kg body weight.



DOSE-RESPONSE RELATIONSHIPS FOR SOMAN, SARIN, AND DFP

Each point represents the mean of at least 10 experiments. Note that in spite of the wide range of doses, the slopes of the curves are nearly identical. Soman is the most potent of the 3 agents, with a range of pressor doses between 10 and 40 ug/kg. Sarin is only slightly less potent, with doses between 15 and 50 ug/kg. In contrast, the threshold dose of DFP is about 300 ug/kg, and a maximal response occurs at 2 mg/kg.

Maximum effects were seen at doses approximating the LD50. The magnitude of the pressor response appears to be limited by death.

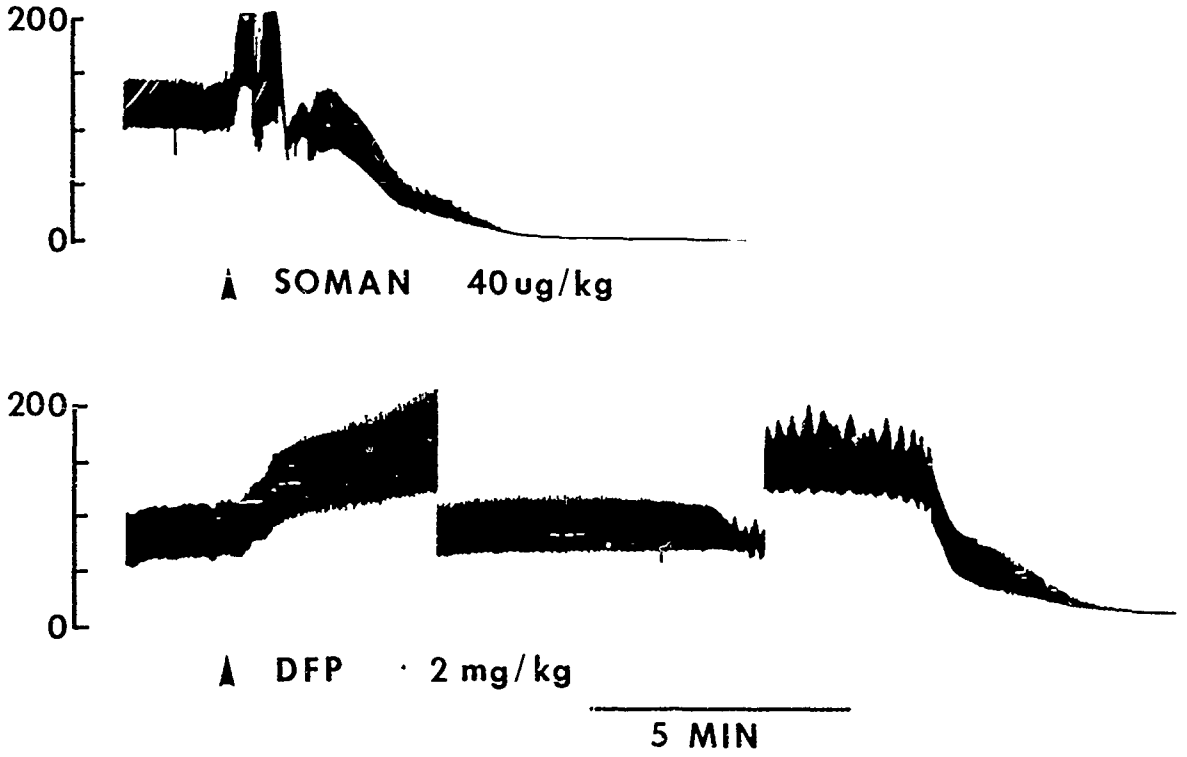


BLOOD PRESSURE RESPONSES TO I.V. INJECTION OF SOMAN, SARIN, AND DFP

The upper tracing depicts the response to 30 ug/kg of soman. Note the virtually immediate increase in blood pressure, which reaches its peak within seconds of injection. Blood pressure occasionally decreases somewhat during the first few minutes and then maintains a relatively stable level for about 30 minutes. Pressure then gradually returns toward normal during the next hour.

The middle tracing shows the pressor response to 40 ug of Sarin. The description of the response is almost identical to that of soman: rapid onset with a duration of about 2 hours at the high doses.

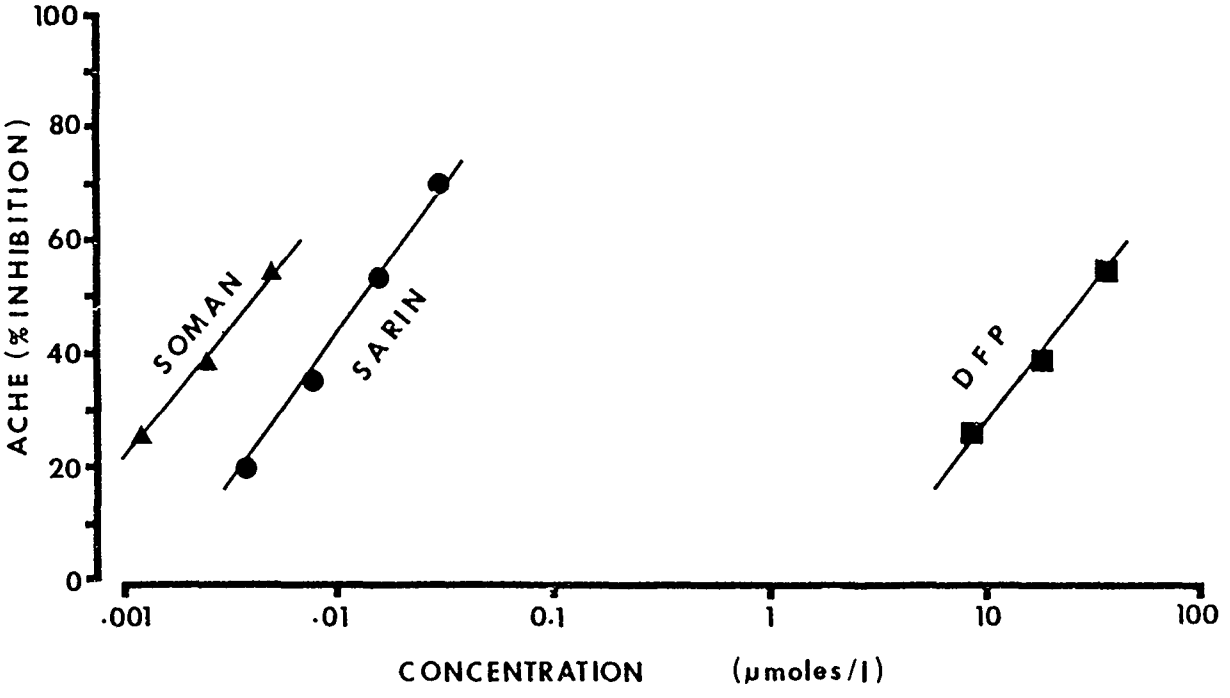
The response to 2000 ug/kg of DFP (bottom tracing) is noticably different from those of soman and sarin. It begins rapidly, but development of the increased blood pressure is gradual and peak elevation does not occur until several minutes after injection.



TIME-COURSE OF DEATH FOLLOWING I.V. INJECTION OF SOMAN AND DFP

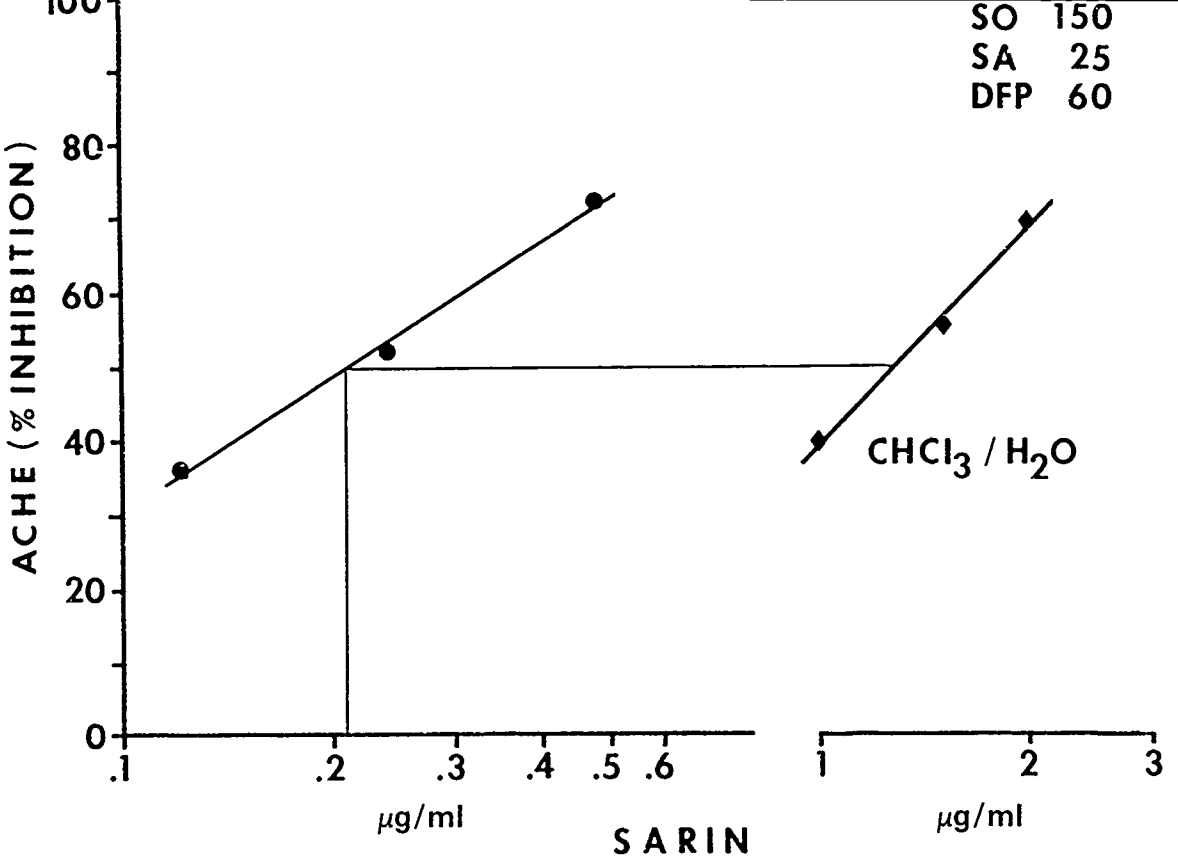
When death occurred following soman or sarin (upper tracing), it almost invariably occurred during the first 5 minutes after injection. In contrast, with DFP (lower tracing), at levels of pressor responses equivalent to those seen with the other agents, few animals died during the first five minutes. Instead, when death occurred it usually was seen 10-15 minutes after drug injection, at a time when the blood pressure appeared to be stable.

(Note: The sensitivity of the recorder was reduced 50% in the middle of the lower tracing).



RELATIONSHIP BETWEEN DRUG CONCENTRATION AND ENZYME INHIBITION.

Note that the slopes of the inhibition curves are similar for the three agents, and that the order of potency follows that seen with the pressor response. This may reflect the K2 bimolecular rate constants, which we calculated to be 10^4 for DFP, but $2x10^7$ for sarin and $8x10^7$ for soman. The in vitro potency of DFP relative to soman and sarin, however, is much less than that observed in vivo. Here, the 50% inhibitory concentration of DFP is nearly 4 orders of magnitude greater than that for soman (15 uM vs 1.7 nM). For the pressor response, the difference was about 30 fold. Sarin was about 4-fold less potent than soman in vitro, but only about 50% less potent in producing a blood pressure rise.



CHLOROFORM/WATER PARTITION COEFFICIENT

The relatively slow development of the pressor response to DFP could be a consequence of a lower lipid solubility or oil:water partition coefficient. Partition coefficients for each of the 3 agents were determined using inhibition of ChE as an end-point. An aqueous solution of agent at known concentration was mixed with chloroform and the inhibitory activity of the aqueous phase was compared to a standard curve. Due to the poor water solubility of DFP, along with the high concentrations required before extraction, a water:chloroform ratio of 4:1 was used.

An example of the analysis is shown above for sarin. On the left is the inhibition curve for sarin alone, at concentrations of 0.12-0.48 μg/ml. On the right is the inhibitory curve of the aqueous phase after extraction with CHCl3. Note that the original solutions contained 1-2 μg/ml of sarin. At a point on this curve giving 50% inhibition, the initial conc of 1.3 μg/ml now gives an inhibition equivalent to 0.21 μg/ml, representing a 6.25-fold loss of activity. Multiplying by 4 to account for the 1:4 ratio gives a partition coefficient of about 25. In a similar way, the partition coefficient for soman was estimated to be 150:1, while that for DFP was only about 60.

CONCLUSION

In summary, intravenous administration of soman, sarin, and DFP increases arterial blood pressure. The order of potencies in producing this response reflects the order of in vitro inhibition of AChE and may reflect the differences in K_2 values. The more gradual rise observed with DFP may reflect the difference in lipid solubility and thus a slower penetration to the site of action.

THE EFFECTS OF ACUTE SOMAN ON SELECTED ENDOCRINE PARAMETERS AND BLOOD GLUCOSE

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ABSTRACT

THE OBJECTIVE OF THIS STUDY WAS TO EVALUATE THE EFFECTS OF A SINGLE CHALLENGE DOSE OF SOMAN (AN IRREVERSIBLE ACETYLCHOLINESTERASE INHIBITOR) ON PLASMA LEVELS OF GLUCOSE, INSULIN, GLUCAGON, NOREPINEPHRINE, EPINEPHRINE, AND CORTICOSTEROIDS. THE RELATIONSHIP OF THESE LEVELS WITH BOTH HYPOTHALAMIC AND ERYTHROCYTE ACETYLCHOLINESTERASE LEVELS WAS ALSO STUDIED.

MALE, SPRAGUE-DAWLEY RATS (300-330 G) WERE GIVEN SUBCUTANEOUS INJECTIONS OF SOMAN AT A CONVULSIVE DOSE (80 UG/KG), A SYMPTOMATIC DOSE WITH SURVIVAL (60 UG/KG), ASYMPTOMATIC DOSE (40 UG/KG), OR SALINE. GROUPS OF SIX RATS FOR EACH DOSE AND TIME PERIOD WERE UTILIZED AND SAMPLES WERE OBTAINED AT 5, 15, 30, AND 60 MINUTES AS WELL AS 2, 4, 8, AND 24 HOURS AFTER SOMAN OR SALINE.

THE CONVULSIVE AND SYMPTOMATIC DOSES OF SOMAN CAUSED PROLONGED DOSE- AND TIME-RELATED INCREASES IN PLASMA LEVELS OF GLUCOSE, EPINEPHRINE, NOREPINEPHRINE, AND CORTICOSTERONE AS WELL AS A DEPRESSION OF INSULIN. THE SYMPTOMATIC DOSE OF SOMAN INDUCED INCREASES IN EPINEPHRINE LEVELS AT THE EARLIER TIME PERIODS WHICH WAS FOLLOWED BY INCREASES IN NOREPINEPHRINE AT LATER TIME PERIODS--AN OBSERVATION WHICH MAY BE DUE TO LONG-TERM STRESS EFFECTS OF SOMAN.

SOMAN INDUCED BOTH TIME- AND DOSE-DEPENDENT DECREASES IN HYPOTHALAMIC ACETYLCHOLINESTERASE WITH THE HIGHEST DOSE INDUCING SIGNIFICANT AND MAXIMAL DECREASES BY 15 MINUTES AND THE LOWEST DOSE PRODUCING MINIMAL EFFECTS ON THIS ENZYME. THE TOXIC SIGNS PRODUCED BY SOMAN TOXICITY, AS WELL AS CHANGES IN NOREPINEPHRINE, EPINEPHRINE, AND CORTICOSTERONE WERE INVERSELY RELATED TO HYPOTHALAMIC ACETYLCHOLINESTERASE LEVELS. A NEAR MAXIMUM DEPRESSION OF ACETYLCHOLINESTERASE ACTIVITY (LESS THAN 2% OF CONTROLS) WAS DETECTED IN ERYTHROCYTES 15 MINUTES AFTER THE ASYMPTOMATIC SOMAN DOSE.

THIS WORK SUPPORTED IN PART BY THE U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND UNDER CONTRACT DAMD-17-83-C-3193.

INTRODUCTION

THE EFFECT OF ACETYLCHOLINESTERASE INHIBITORS ON ENDOCRINE PARAMETERS HAVE BEEN ELUTED TO BY A FEW STUDIES WHICH REPORTED HYPERGLYCEMIA AND INCREASED GLUCOCORTICOID LEVELS AFTER TOXIC DOSES OF PESTICIDES IN EXPERIMENTAL ANIMALS. HYPERGLYCEMIA HAS BEEN REPORTED IN RATS AFTER PARATHION AND SEVEN (1). THE ADRENALS MAY BE INVOLVED SINCE ADRENALECTOMY ABOLISHED THE HYPERGLYCEMIA (2). TOXIC DOSES OF ANTI-CHOLINESTERASE INSECTICIDES ALSO PRODUCE INCREASES IN CORTICOSTERONE LEVELS IN ADDITION TO HYPERGLYCEMIA IN RATS (3).

THE ROLE OF THE HYPOTHALAMUS IN THE REGULATION OF ENDOCRINE FUNCTION AND BLOOD GLUCOSE HAVE RECEIVED MUCH ATTENTION. ACETYLCHOLINE INJECTED INTO THE VENTRAL MEDIAL HYPOTHALAMUS OF RABBITS CAUSES HYPERGLYCEMIA AND INCREASED GLUCAGON LEVELS; SIMILAR EFFECTS ARE SEEN AFTER ELECTRICAL STIMULATION (4, 5). LESIONS OF THE VENTRAL MEDIAL HYPOTHALAMUS RESULT IN HYPERINSULINEMIA, HYPERPHAGIA, AND OBESITY IN RATS (6). ACETYLCHOLINE PLACED INTO THE HYPOTHALAMUS ALSO CAUSES A RELEASE OF ACTH FROM THE PITUITARY (7).

SOMAN, AN IRREVERSIBLE ACETYLCHOLINESTERASE INHIBITOR, INCREASES THE AMOUNT OF ACETYLCHOLINE AT MUSCARINIC AND NICOTINIC NEURONS WHICH CAN POTENTIALLY CAUSE CHANGES IN THE NEUROLOGICAL INPUT INTO ENDOCRINE GLANDS. THE PURPOSE OF THIS STUDY WAS TO EVALUATE THE EFFECTS OF GRADUATED ACUTE DOSES OF SOMAN ON THE PLASMA LEVELS OF GLUCOSE, INSULIN, GLUCAGON, NOREPINEPHRINE, EPINEPHRINE AND CORTICOSTEROIDS AND TO EVALUATE THE RELATIONSHIP BETWEEN THESE AND THE LEVELS OF ACETYLCHOLINESTERASE ACTIVITY IN THE HYPOTHALAMUS.

METHODS

1. MALE SPRAGUE-DAWLEY RATS WEIGHING 300-330 G WERE GIVEN SOMAN S.C. IN THE FOLLOWING DOSES:

   80 µG/KG (CONVULSIVE DOSE)

   60 µG/KG (SYMPTOMATIC DOSE)

   40 µG/KG (ASYMPTOMATIC DOSE)

2. GROUPS OF SIX RATS FOR EACH SOMAN DOSE AND A GROUP OF SIX RATS DOSED WITH SALINE WERE DECAPITATED AND TRUNK BLOOD WAS OBTAINED FOR ASSAYS AT 5, 15, 30, AND 60 MIN AND AT 2, 4, 8, AND 24 HOURS AFTER SOMAN AND SALINE DOSES. THE HYPOTHALAMUS WAS DISSECTED AS DESCRIBED BY GLOWINSKI AND IVERSON (8). SAMPLES FOR THE DETERMINATION OF ERYTHOCYTE ACETYLCHOLINESTERASE ACTIVITY WERE OBTAINED FROM PACKED CELLS FOLLOWING CENTRIFUGATION.

3. THE 80 UG/KG DOSED RATS WERE NOT STUDIED BEYOND 60 MINUTES.

4. INSULIN, GLUCAGON, AND CORTRICOSTERONE PLASMA LEVELS WERE DETERMINED UTILIZING COMMERCIALLY AVAILABLE RADIOIMMUNOASSAY KITS MODIFIED FOR USE IN THIS STUDY. NOREPINEPHRINE AND EPINEPHRINE WERE DETERMINED BY UTILIZING A CATECHOLAMINE ANALYZER (HPLC WITH AN ELECTROCHEMICAL DETECTOR; BIOANALYTICAL SYSTEMS, INC). A MODIFICATION OF THE RADIOMETRIC ASSAY OF STERRI AND FONNUM (9) WAS USED FOR THE DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY OF THE HYPOTHALAMUS AND ERYTHOCYTES. GLUCOSE LEVELS WERE OBTAINED USING THE BECKMAN GLUCOSE ANALYZER II.

5. THE INTENSITY OF THE TOXIC SIGNS WERE DETERMINED USING ASSIGNED SCORES FROM 1 TO 5 WITH FIVE BEING THE MOST INTENSE TOXIC SIGN (INCLUDING TONIC SEIZURES).

6. THE DATA WERE ANALYZED BY ANALYSIS OF VARIANCE FOR EACH TIME INTERVAL AND THE MEANS WERE COMPARED UTILIZING THE NEWMAN-KEULS RANGE TEST. WHERE APPROPRIATE, CORRELATION COEFFICIENTS WITH LEVELS OF SIGNIFICANCE WERE OBTAINED BY CALCULATING THE PEARSON CORRELATION COEFFICIENT FOR PARAMETRIC DATA AND THE SPEARMAN COEFFICIENT FOR NON-PARAMETRIC DATA.

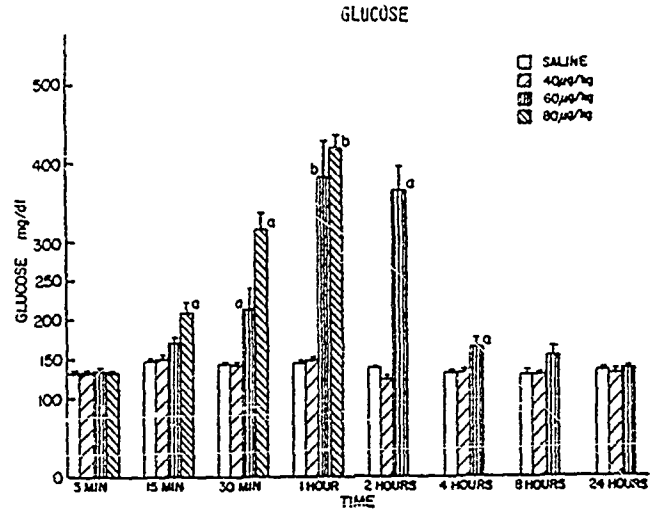

Figure 1. The Effect of Somsn on Plasma Glucose Levels

[a]Significantly different from all groups in the same time period.

[b]Significantly different from saline and 40 ug/kg groups.

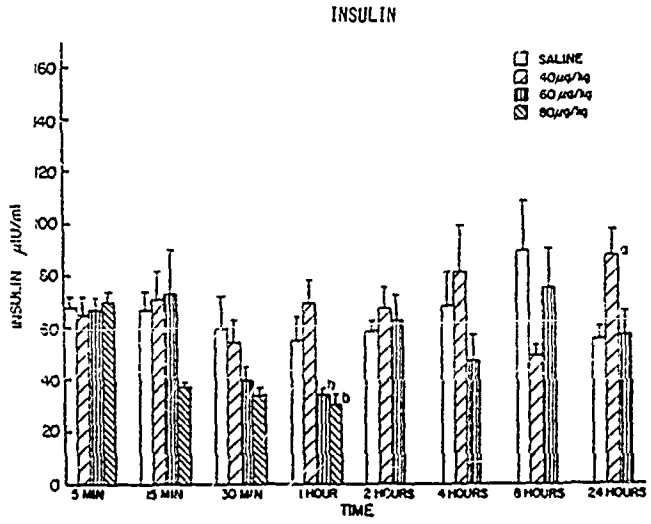

Figure 2. The Effect of Somsn on Plasma Insulin Levels.

[a]Significantly different from all groups in the same time period.

[b]Significantly different from saline and 40 ug/kg groups.

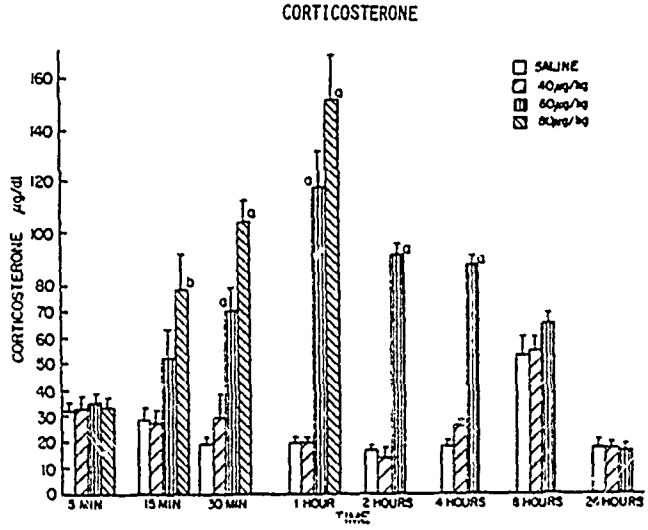

Figure 3. The Effect of Somsn on Plasma Corticosterone Levels

[a]Significantly different from all groups in the same time period.

[b]Significantly different from saline group.

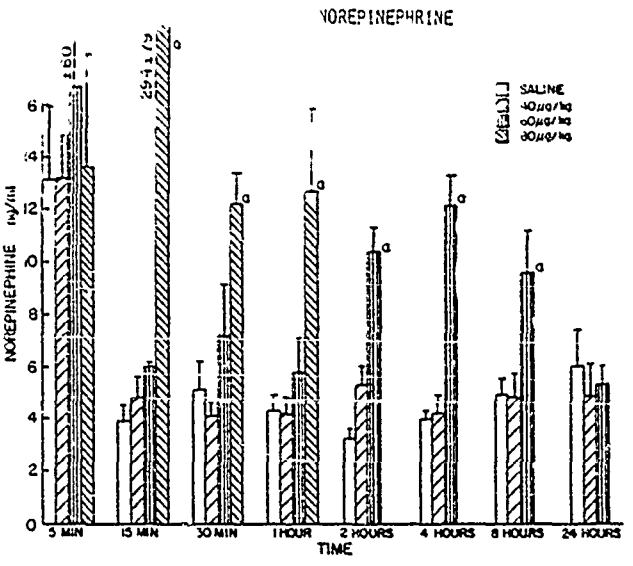

Figure 4. The Effect of Soman on Plasma Norepinephrine Levels

[a]Significantly different from all groups in the same time period.

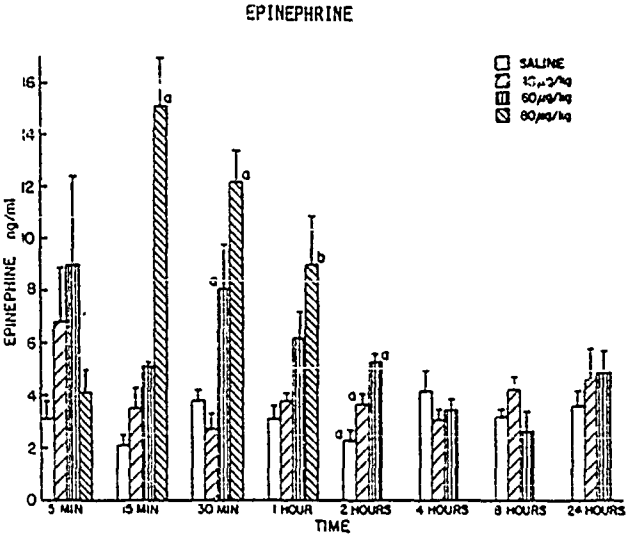

Figure 5. The Effect of Soman on Plasma Epinephrine Levels

[a]Significantly different from all groups in the same time period.

[b]Significantly different from saline and 40 ug/kg groups.

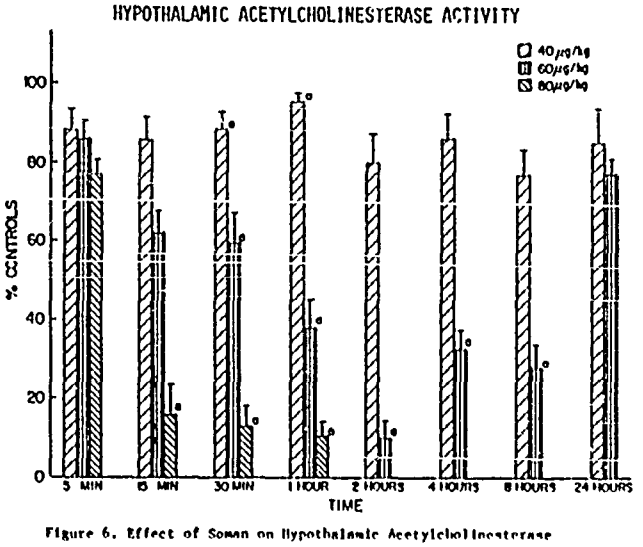

Figure 6. Effect of Soman on Hypothalamic Acetylcholinesterase

[a]Significantly different from all groups in the same time period.

Effect of Soman on Erythrocyte Acetylcholinesterase
(% Control ± SEM)

Dose Soman	Time							
	5 min	15 min	30 min	60 min	2 hr	4 hr	8 hr	24 hr
40 ug/kg	24.5 ±5.6	1.6[a] ±0.37	2.6 ±0.32	12.3[a] ±4.4	3.9 ±2.4	3.2 ±2.8	15.9 ±7.6	13.9 ±6.7
60 ug/kg	15.8 ±4.3	0.58 ±0.25	0.67 ±0.37	0.73 ±0.31	-0-	3.5 ±2.0	3.8 ±0.44	2.5 ±1.1
80 ug/kg	1.8[a] ±0.6	0.45 ±0.22	2.0 ±1.4	0.37 ±0.24	+	+	+	+

+ Animals died prior to 2 hour time interval.

[a]Significantly different from other groups in same time period.

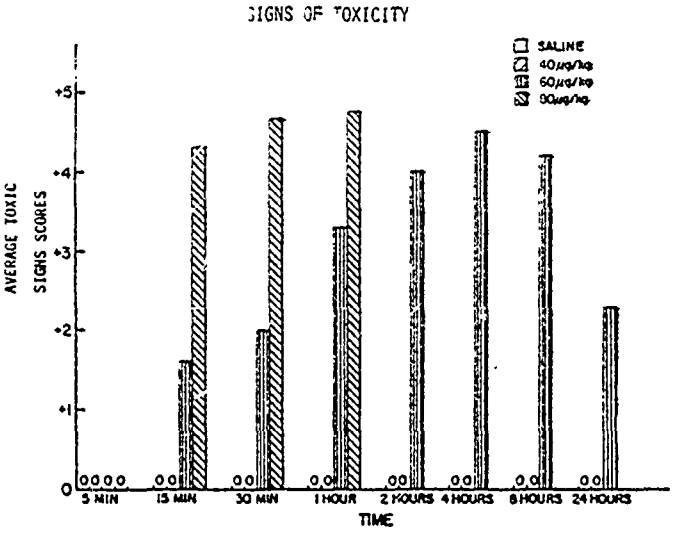

Figure 7. TOXIC SIGNS SCORES FOR SOMAN DOSES

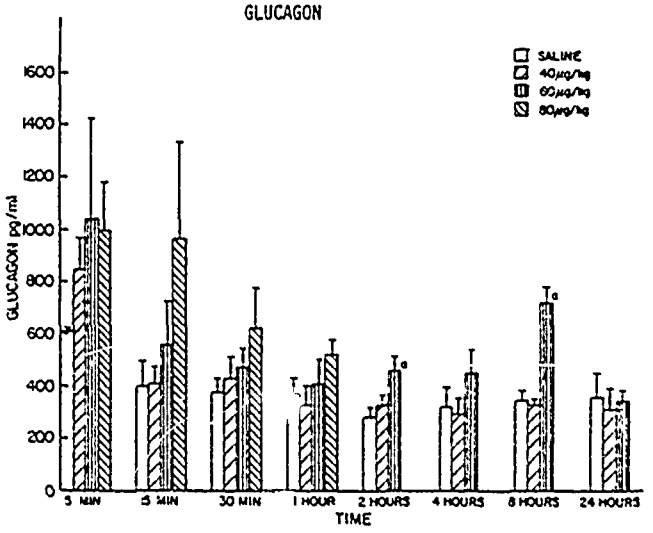

Figure 8. Effect of Soman on Plasma Glucagon Levels

[a]Significantly different from saline and 40 ug/kg groups.

DISCUSSION

1. ACUTE CONVULSIVE AND SYMPTOMATIC DOSES OF SOMAN CAUSE PROLONGED DOSE- AND TIME-RELATED CHANGES:

   ↑ EPINEPHRINE

   ↑ NOREPINEPHRINE

   ↑ GLUCOSE

   ↑ CORTICOSTERONE

   ↓ INSULIN

   THESE RESULTS INDICATE THAT THE ENDOCRINE RESPONSE TO SOMAN TOXICITY INVOLVES THE ADRENAL MEDULLA AND CORTEX, THE SYMPATHETIC NERVOUS SYSTEM AND THE ENDOCRINE PANCREAS.

2. THE DEPRESSED HYPOTHALAMIC ACETYLCHOLINESTERASE ACTIVITY, ALONG WITH OTHER AREAS OF THE BRAIN, MAY PLAY AN IMPORTANT ROLE IN THE INCREASED LEVELS OF CORTICOSTERONE, EPINEPHRINE, AND NOREPINEPHRINE SINCE SIGNIFICANT, NEGATIVE CORRELATION COEFFICIENTS WERE OBTAINED FOR THE 80 AND 60 UG/KG SOMAN DOSES AT THE 15, 30, AND 60 MIN TIME INTERVALS.

3. THE HYPERGLYCEMIA WHICH ACCOMPANIES SOMAN TOXICITY APPEARS TO BE A RESULT OF ELEVATED CATECHOLAMINES, CORTICOSTERONE, AND A DEPRESSED INSULIN RESPONSE TO GLUCOSE. IF GLUCAGON PLAYS A ROLE IN THE HYPERGLYCEMIA, THESE RESULTS INDICATE THAT IT IS A TRANSIENT ONE.

4. MOST OF THE CHANGES IN GLUCOSE, GLUCAGON, CORTICOSTERONE, INSULIN, AND CATECHOLAMINES ARE CONSISTENT WITH KNOWN EFFECTS OF DIRECT ACETYLCHOLINE STIMULATION OF THE HYPOTHALAMUS. HOWEVER, THESE CHANGES COULD ALSO BE EXPLAINED AS AN INDIRECT RESPONSE TO STRESS OF PERIPHERAL ORIGIN.

5. THERE ARE SOME INDICATIONS THAT DURING THE EARLY TIME INTERVALS AT LEAST PART OF THE ENDOCRINE CHANGES MAY RESULT FROM SOMAN'S EFFECT AT THE HYPOTHALAMIC LEVELS. THE ELEVATED EPINEPHRINE AND CORTICOSTERONE LEVELS FOR THE 60 UG/KG DOSE AT 30 MIN OCCUR WHEN THE TOXIC SIGN SCORES FOR THESE ANIMALS ARE LOW.

6. THE CONTRIBUTIONS OF DIRECT CENTRAL STIMULATION AND INDIRECT PERIPHERAL STIMULATION TO THE HORMONAL RESPONSES ARE NOW BEING INVESTIGATED.

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EFFECTS OF ORGANOPHOSPHATE CHOLINESTERASE INHIBITORS ON RENAL FUNCTION

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ABSTRACT

Both diisopropylfluorophosphate (DFP) and soman alter renal function apparently by mechanisms unrelated to inhibition of cholinesterase. The DFP effects have been prompt increase in urine flow which reverses to the control state by 24 hours after the administration of a single, effective dose in the unanesthetized rat. Doses in the range of 1 to 4 mg/kg were effective. The urine produced was of relatively low osmolality, and contained significant quantities of glucose and protein. Sodium excretion was increased, but potassium excretion was not. DFP also affected the transport of organic anions by the kidney and this observation was true whether the animals were pretreated with DFP or it was added to fresh renal cortex slices under in vitro conditions. These effects apparently were not mediated through alterations in renal hemodynamics, at least as judged by the usual measurements of renal blood flow, glomerular filtration rate, etc. Also, a surfeit of anti-diuretic hormone did not alter this response.

Soman produced similar effects, although these were seen less readily in the Sprague-Dawley rat than in the Fischer 344 rat. The Fischer rat was more sensitive to the effects of soman than the Sprague-Dawley animal and, not surprisingly the effects on the kidney were seen only when the rats did not show gross toxic effects to this cholinesterase inhibitor. In any event, approximately the same effects on renal function were observed with soman in the Fischer rat as were produced by DFP in either the Fischer or the Sprague-Dawley rat. In anesthetized rats, soman produced alterations in renal hemodynamics (increased blood flow, increased glomerular filtration rate) in some animals. Mean values, however, were not significantly different from controls.

Whatever the nature of these effects on renal function, they apparently were not related to inhibition of renal cholinesterase. Both soman and DFP affect CNS cholinesterase equally well, but a clear correlation between cholinesterase inhibition in the kidney and the effects on renal function were not observed. Cholinesterase inhibition in the kidney was variable despite dramatic effects on renal function.

Both soman and DFP inhibit Na^+, K^+-activated, Mg^{++}-dependent ATPase significantly in the same doses that alter renal function. No effects were observed on the Mg^{++}-ATPase. Changes also were observed in non-protein sulfhydryl groups in the kidney, but not in protein bound sulfhydryls.

This work supported in part by the U. S. Army Medical Research and Development Command under contract DAMD 17-82-C-2220.

INTRODUCTION

The clearest evidence to suggest a role for the automatic nervous system in the regulation of renal function may well have come from the studies of Barajas and colleagues (reviewed by Barajas, 1978). These studies, mostly morphological and histochemical, have yielded evidence of an extremely rich adrenergic innervation of the kidney in most species and an interesting relationship between acetylcholinesterase and that adrenergic innervation. Several investigators (reviewed by DiBona, 1977) have presented physiological data that suggest a direct role of the sympathetic nerves in renal tubular sodium reabsorption, i.e. not mediated through alterations of glomerular filtration rate (GFR) or renal blood flow (RBF).

Direct effects of cholinergic agents on renal function and transport appear to be more complicated, however. Earlier work by Carter, Vander and others demonstrated that there were effects of cholinergic agents, but Earley and Friedler suggested that the increase in urine flow due to acetycholine, for example, was due to an increase in medullary blood flow, thus resulting in a washout of the medullary interstitium. Hence, these early studies suggested that whatever effects were exerted on renal function by cholinergic agents were related to increases in renal blood flow because cholinergic agents are vasodilators. The work of Carriere et al. called this interpretation into question, since these workers found no change in medullary blood flow after administration of acetylcholine or other vasodilators. These studies utilized the krypton washout technique. Further Avrunin and Carter found no washout of the cortico-medullary sodium gradient in the rat kidneys after administration of bethanechol.

The possibility of direct effects of cholinomimetics on renal function may exist (Carter, 1971). Carter's group demonstrated that acetycholine and propionylcholine could alter renal slice function after direct addition of these compounds. When used in high concentrations, increases in tissue sodium was increase. Although it is by no means clear how these data fit into the overall scheme of a cholinomimetic-induced diuresis, they do demonstrate a direct action of these compounds on renal cell electrolyte balance.

Effects of cholinesterase inhibitors on renal function or on renal slice electrolyte balance have not been studied. Cholinesterase inhibitors might have effects on renal function similar to those of cholinomimetics, or might act through direct mechanisms related to the inherent reactivity of these active phosphorylating agents.

L. Barajas, Innervation of the renal cortex. Fed. Proc., 37 (1978) 1192.

G. F. DiBona, Neurogenic regulation of renal tubular sodium reabsorption. Am. J. Physiol., 233 (1977) F73.

M. K. Carter, Renal electrolyte changes and vasoactive agents, in J. W. Fisher and E. J. Cafruny (Eds.), Renal Pharmacology, Appleton-Century-Crafts, New York, 1971, pp. 43-65.

MATERIALS AND METHODS

Male Sprague-Dawley rats (approx. 200-250 g) from Charles River Laboratories, Wilmington, MA, Blue Spruce Farms, Altamont, NY, or Sasco, Inc., Omaha, NE and Fischer 344 rats from Charles River or Harlan Labs were used in these studies. Soman and DFP were administered subcutaneously. Prior to and during the experiments, the animals were housed in a room with automatic 12 h light-dark cycles and a controlled temperature of 25°C.

For the experiments with unanesthetized animals, the rats were housed individually in stainless steel metabolism cages for 24 hours before any urine collections. Subsequently 2 or 3 control days were allowed before injection of the DFP. On each control and experimental day, food was not available during the first 6 h (08.30-14:30 h), and water was available and libitum. Powdered Purina Rat Chow was provided during the remaining 18 h of each day. Three urine samples were collected on each day: 0-2.5 h (08:30-11:00 h), 2.5-6.0 h and 6-24 h. Water and food consumption were determined for each 24-h period, and the animals were weighed daily.

Urine samples were anaylzed for volume, osmolality (freezing point depression), sodium and potassium (flame photometry). Excretion of glucose, protein and blood were assessed qualitatively with BiliLabStix (Ames).

Cholinesterease activity was assessed by the method of Ellman et al. Aliquots of brain or kidney homogenates (8-16 mg tissue) were added to cuvettes containing 0.1 M phosphate buffer and 5,5'-dithobis-2-nitrobenzoic acid (DTNB) reagent (0.01 M in 0.1 M phosphate buffer, pH 7.0). Twenty microliters of acetylthiocholine iodide (0.075 M) or butyrylthiocholine iodide (0.075 M) were added to the sample cuvette. The absorbance was measured at 412 nm with a Beckman-2600K spectrophotometer or a Gilson spectrophotometer and changes in absorbance were recorded. In studies of the in vitro inhibitory effects of DFP, the same procedures were used except that various concentrations of DFP were added directly to the reaction mixture.

In the experiments where hemodynamic parameters were measured the rats were anesthetized with pentobarbital (60 mg/kg i.p.). Inulin clearance was measured to assess GFR, and total RBF was monitored with an electromagnetic flow meter (Carolina Medical Electronics, Inc.). One jugular vein and one carotid artery were cannulated with polyethylene tubing (PE 50), and both ureters were cannulated with PE 10 tubing. A saline solution (0.15 M sodium chloride) containing [^{14}C] inulin was infused i.v. Blood samples were taken periodically over the course of the experiment. Urine was collected at 15 or 20 min intervals with 2 control urine samples collected before the SQ injection. DFP was administered in doses of 1 mg/kg, at 0.5 h intervals. Inulin in urine and plasma was assayed by liquid scintillation spectrometry. Although blood flow was measured continuously, the values at the mid-point of each urine collection were used for statistical analyses and data presentation. The 2 values for control and each dose of DFP were averaged, as were the urine flows and inulin clearances.

Statistical analyses were performed with Student's t-test or an Analysis of Variance with the significance of the differences between means determined by Student-Neuman-Keuls test [26]. Probability values of $P < 0.05$ were accepted as significant.

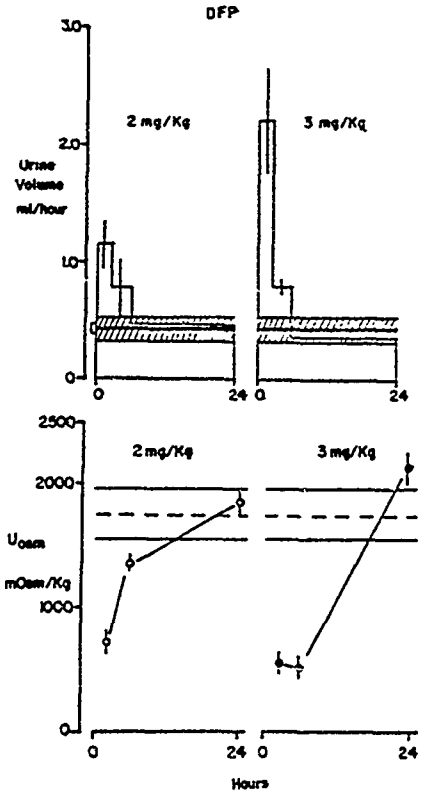

Figure 1.a. Effect of 2 doses of DFP on urine volume and osmolality in male, Sprague Dawley rats.

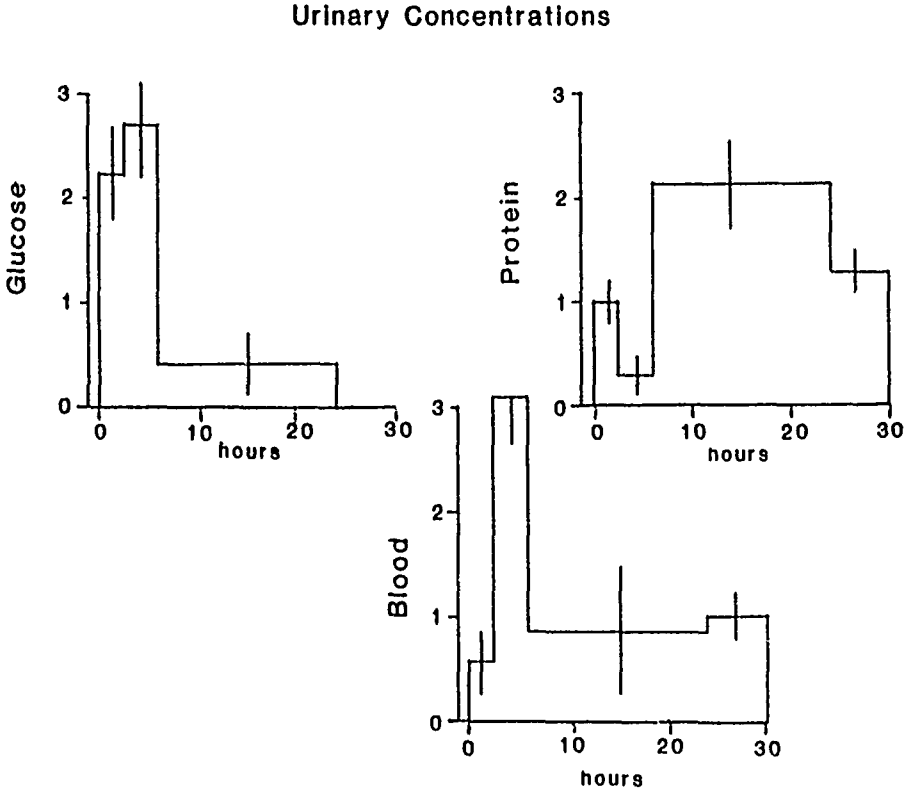

Figure 1.b. Effect of 3 mg DFP/kg on urinary excretion of glucose, protein and blood by male, Sprague Dawley rats.

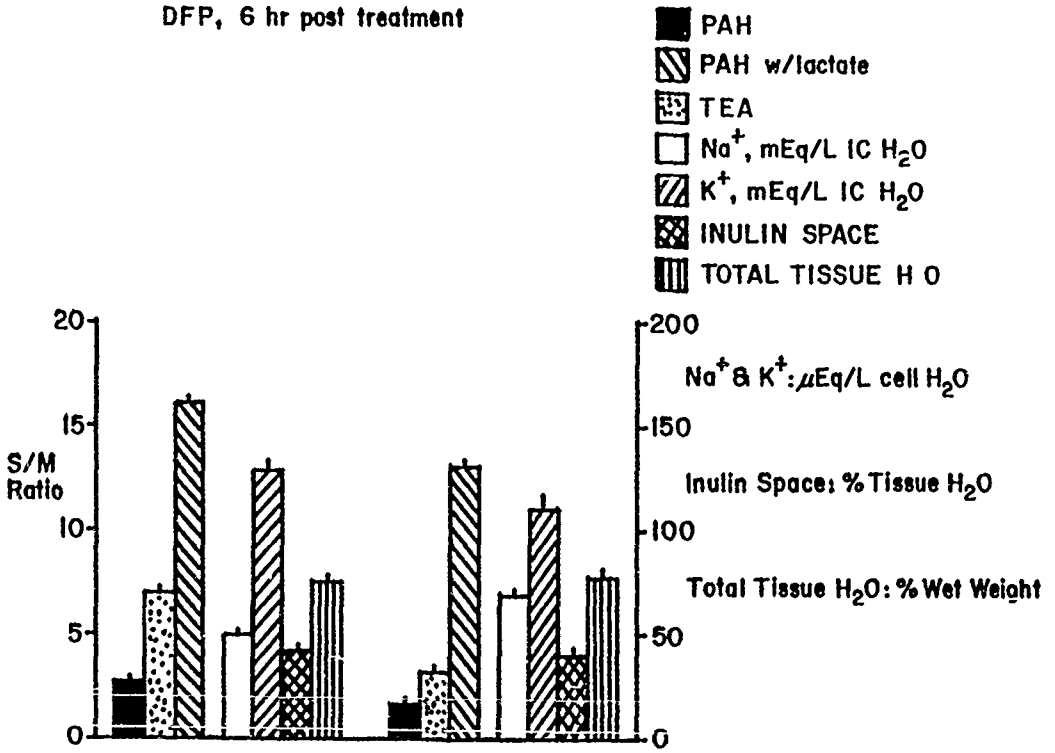

Figure 2. Effect of DFP, 3 mg/kg, on renal slice organic ion accumulation and renal electrolyte and water balance. Both the reduced PAH and TEA uptakes are statistically significant ($P < .05$).

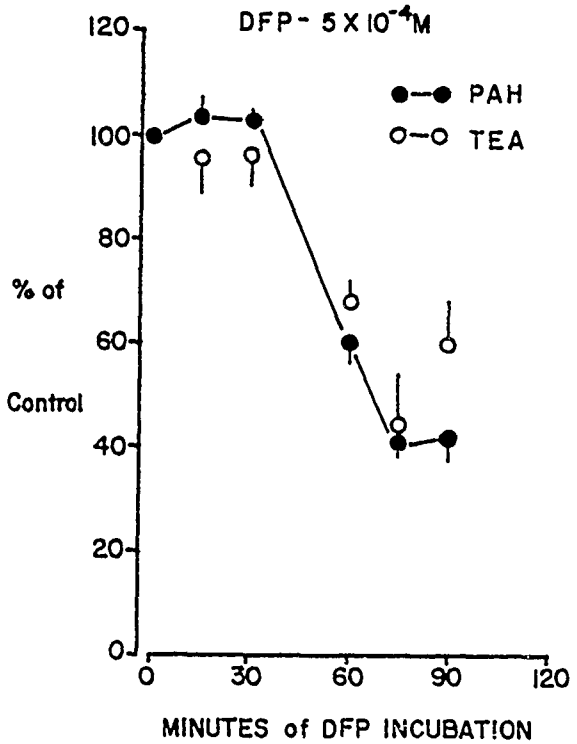

Figure 3. DFP added in vitro to renal cortex slices significantly decreased PAH and TEA accumulation after 60 minutes of preincubation.

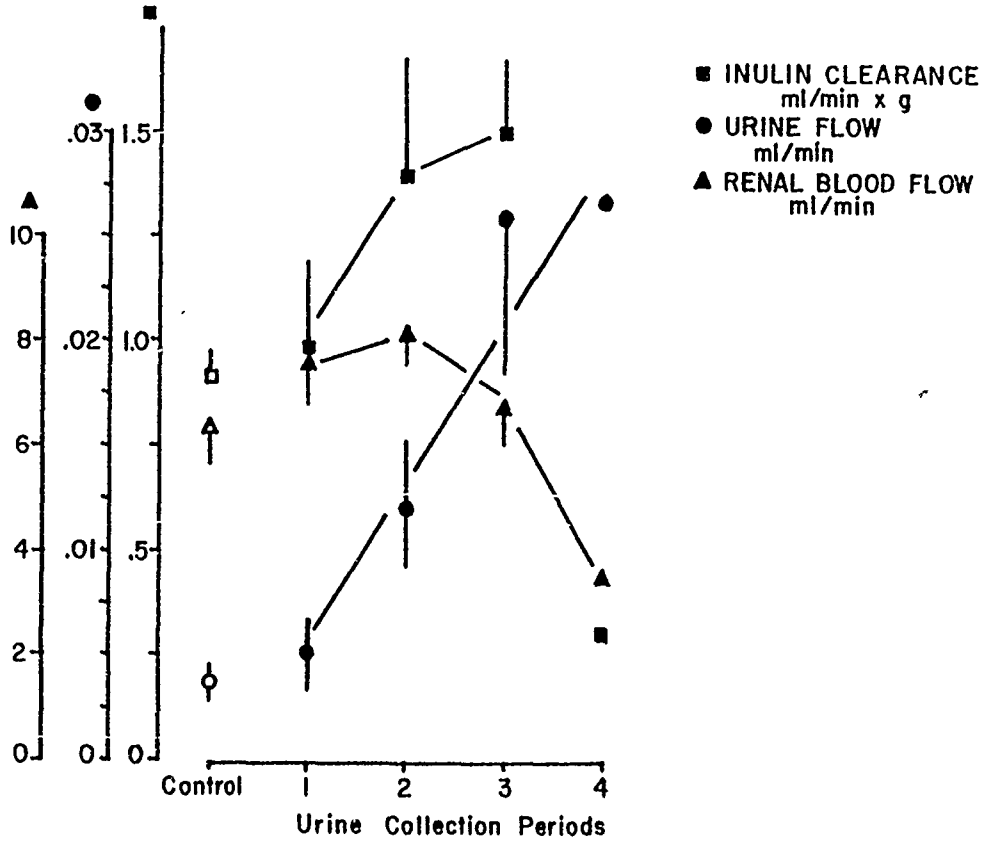

Figure 4. In anesthetized rats, the effects on urine flow rate do not correlate with changes in renal blood flow or glomerular filtration rate.

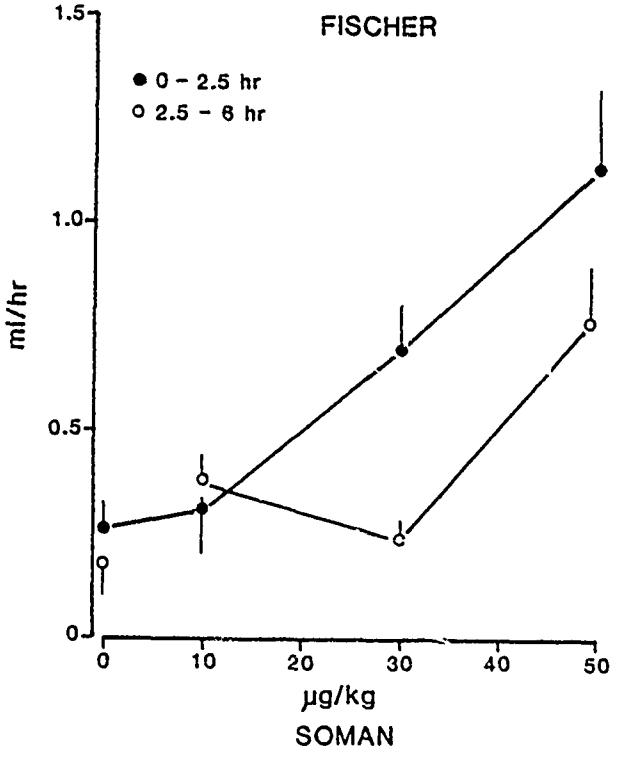

Figure 5. As judged by changes in urine flow, soman alters renal function in a manner similar to that for DFP. These effects were seen in the Fischer 344 rat.

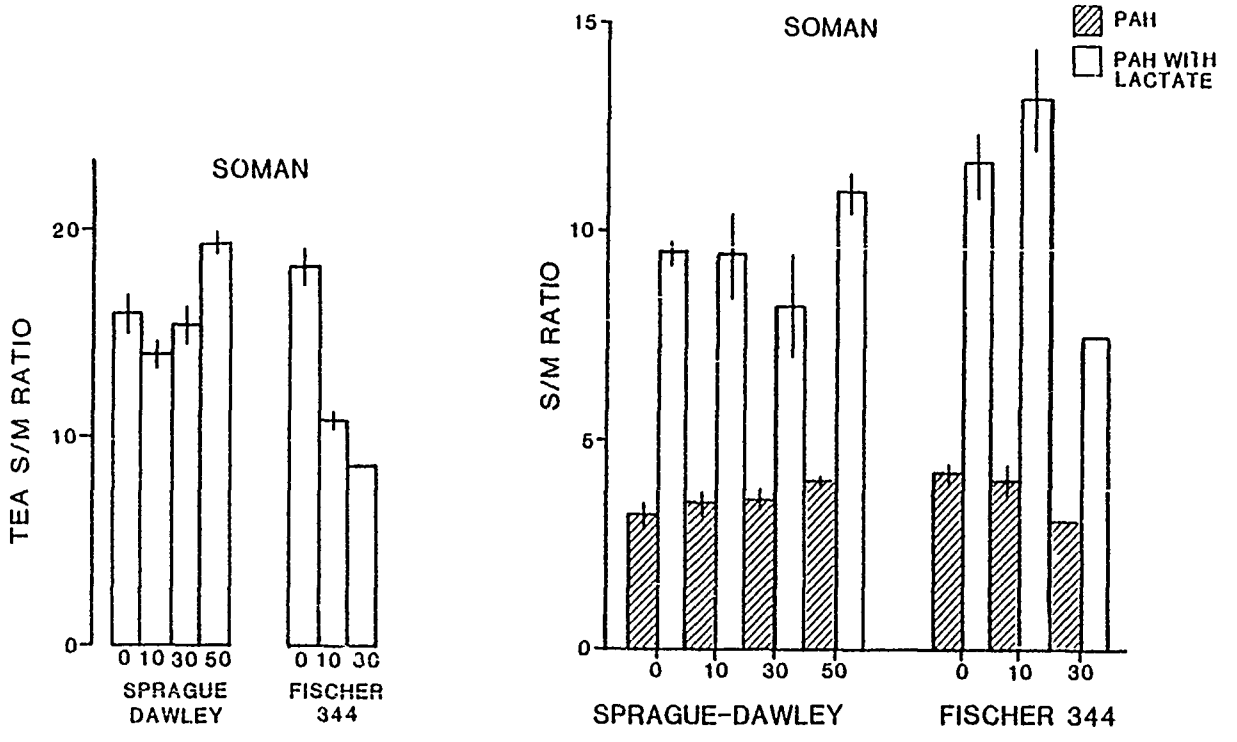

Figure 6. The sensitivity of the Fischer strain to soman also was observed with renal slice organic ion accumulation 6.0 hours after pretreatment.

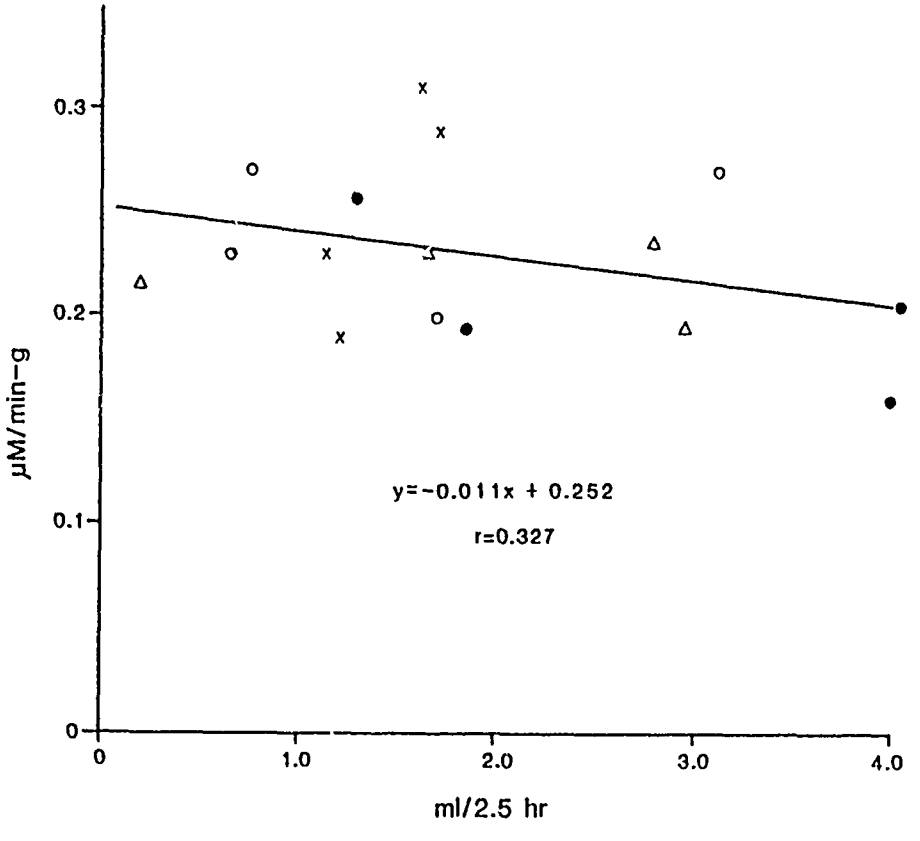

Figure 7. The effects of the organophosphate compounds on urine flow do not correlate with inhibition of renal cholinesterase. These experiments were performed both in the Sprague Dawley and the Fischer strains with DFP and soman.

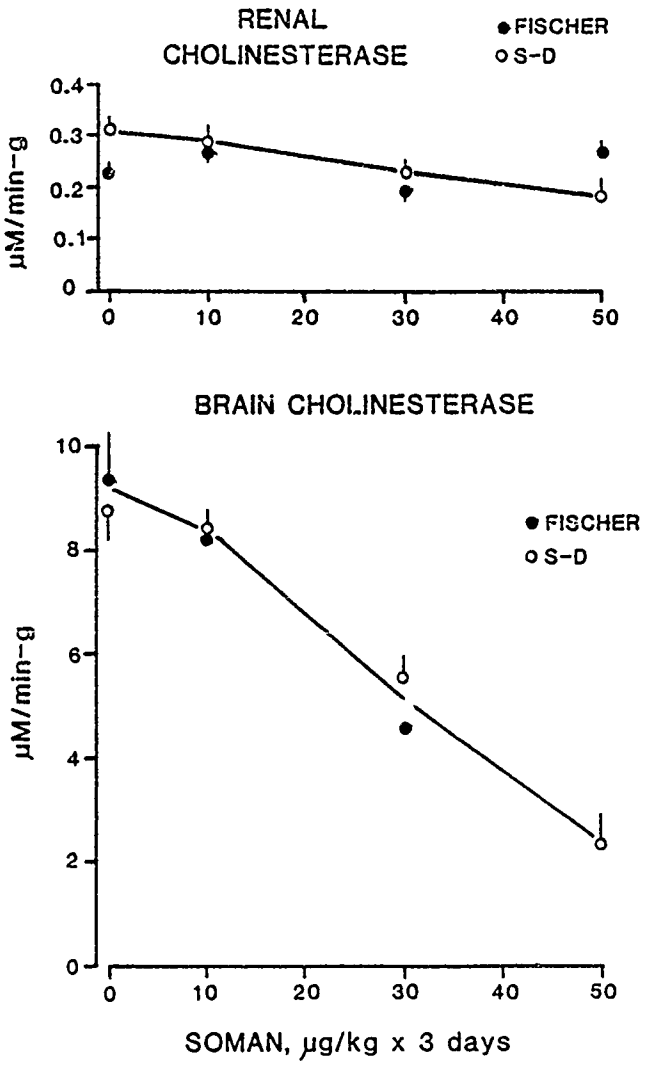

Figure 8. Even with maximally effective doses of soman, renal cholinesterase is inhibited about 30% in the most sensitive strain. Brain cholinesterase is inhibited by about 75%.

TABLE 1

Effects of DFP and soman on renal cortex microsomal ATPase activities. Data are μM Pi/hr/mg protein. The significant depressions of the Na^+ K^+ activated enzyme occur at doses which alter renal function in the rat. Enzyme activities were measured 2-5 hours after pretreatments.

	ATPase Activity	
	Mg^{++}	Na^+-K^+
Control	31.1	59.5
DFP 2.0 mg/kg	31.6	44.3
DFP 2.5 mg/kg	29.8	37.7
Soman 30μg/kg	29.2	48.1
Soman 50μg/kg	31.3	47.7

CONCLUSIONS

1. Organophosphate cholinesterase inhibitors can alter renal function in the rat apart from effects on renal hemodynamics.

2. Renal slice accumulation of organic ions can be inhibited by organophosphate compounds both in vitro and after pretreatment.

3. Alterations in renal function and transport caused by the organophosphate compounds does not correlate with inhibition of renal cholinesterase.

4. Inhibition of Na^{++}-K^{+} activated ATPase correlates with changes in renal function.

PHYSIOLOGICAL RESPONSE OF RATS TO ACUTE IN ALATION EXPOSURES OF HYDROGEN CYANIDE

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ABSTRACT

Hydrogen cyanide (HCN) is a rapidly acting toxic compound. In this series of experiments, Fischer 344 male rats received square wave inhalation exposures to preset atmospheric concentrations of HCN mixed with air such that oxygen deprivation did not contribute to the toxicity. The exposure chambers were designed such that only the heads of the rats were exposed. All animals surviving exposure were observed for at least 14 days.

EC_{50} values (the concentration of HCN necessary to incapacitate 50% of the animals during the exposure) and LC_{50} values (the concentration of HCN needed to kill 50% of the animals during the exposure and a 24 hour post-exposure period) were determined for exposure times ranging from 1-10 minutes. These LC_{50} values were compared to previously determined values for 20, 30, and 60 minute exposures. At concentrations close to the LC_{50} values, both blood pressure and heart rates were measured. Blood cyanide and arterial plasma histamine levels were monitored before, during, and post-exposure.

Following exposure, many completely incapacitated animals (no righting reflex) were observed to exhibit convulsive seizures prior to regaining consciousness. Most deaths occurred during the exposures or within 24 hours. Some animals, however, did not recover immediately but continued to lose considerable weight (up to 56%) over many days (up to 38) before dying.

OBJECTIVE

To determine the physiological effects and incapacitating and lethal levels of short term acute inhalation hydrogen cyanide exposures

METHODS

ANIMAL EXPOSURES

- 5-6 male Fischer 344 rats per test
- Exposed head only
- Square wave exposures

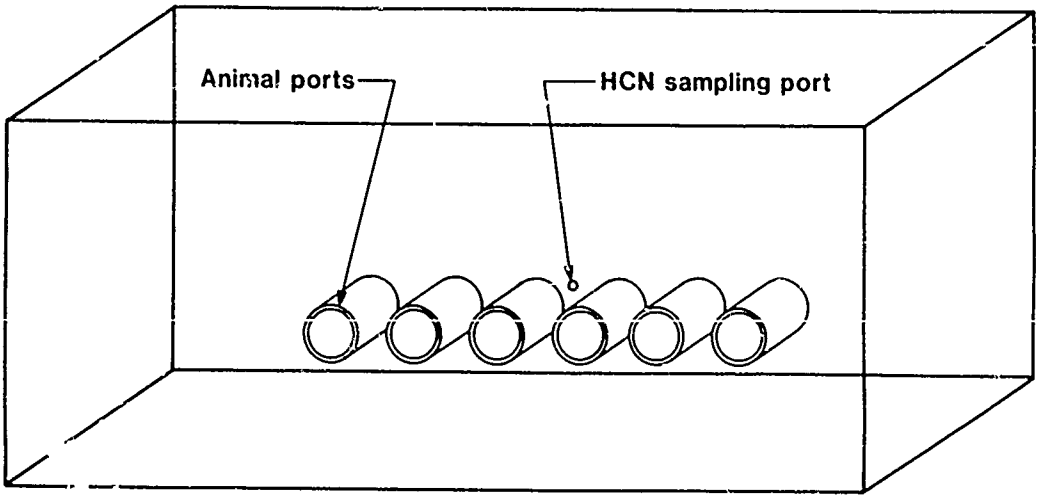
BIOLOGICAL MEASUREMENTS

- Blood pressure
- Heart rate
- Blood cyanide
- Histamine

ENDPOINTS

- EC_{50}: Incapacitation (righting reflex)
- LC_{50}: Lethality (exposure time plus 24 hours)

EXPOSURE SYSTEMS



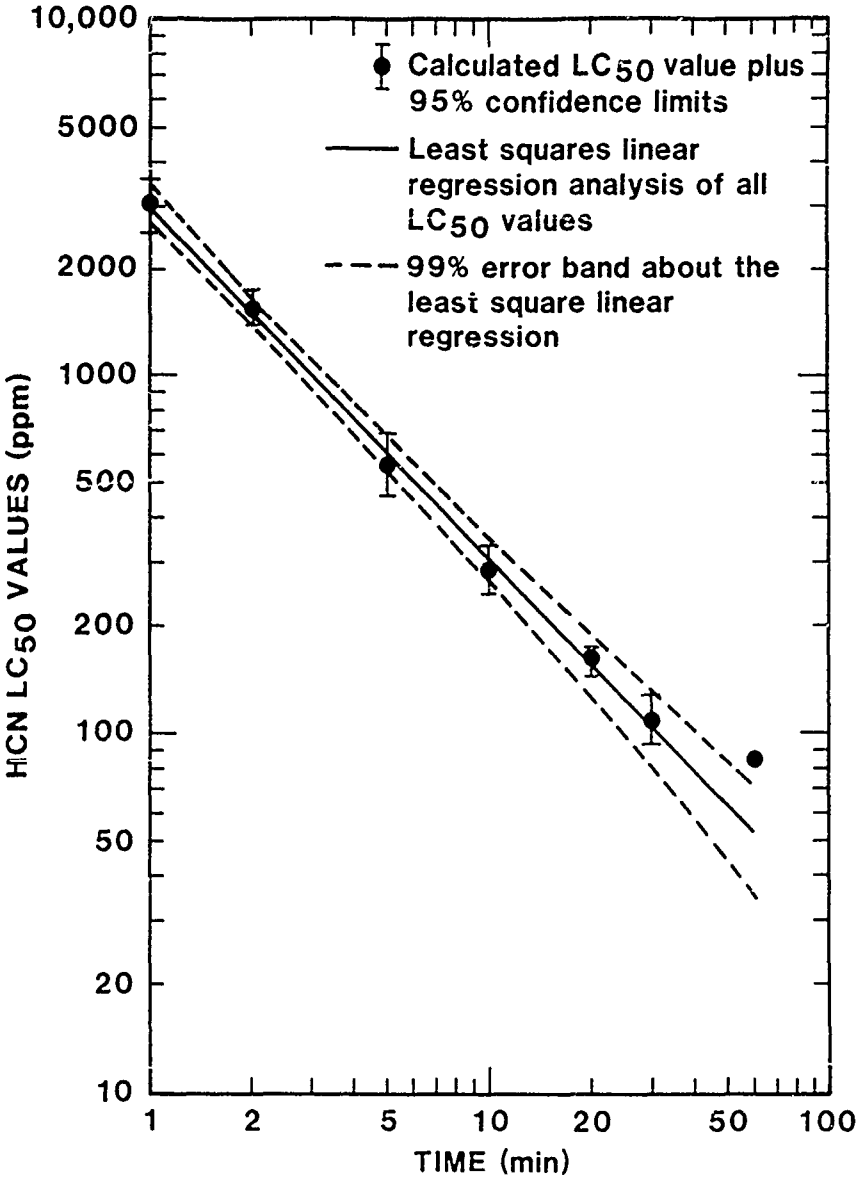
Static exposure:	Dynamic exposure:
Volume - 200 liters	Volume - 4.5 liters
Time - 2-60 minutes	Time - 1 minute

RESULTS
Lethality and Incapacitation Values

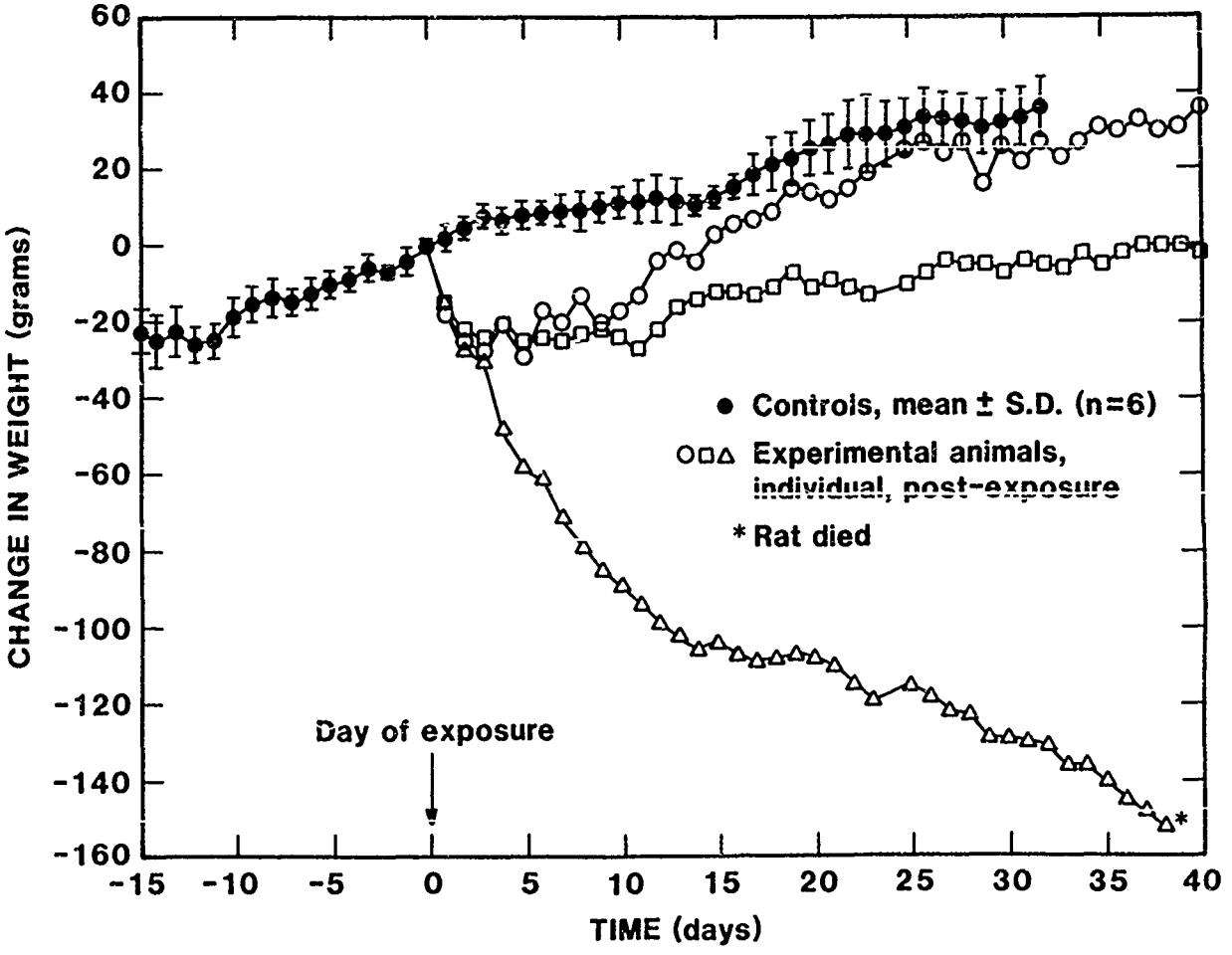
Time (min)	LC_{50} (ppm)	EC_{50} (ppm)
1	3010 (2520-3600)*	1710 (1220-2390)
2	1570 (1390-1770)	1050 (830-1330)
5	570 (460-710)	390 (310-490)
10	290 (250-340)	170 (150-190)
20	170 (160-175)	--
30	110 (95-130)	--
60	86	--

*95% confidence limits

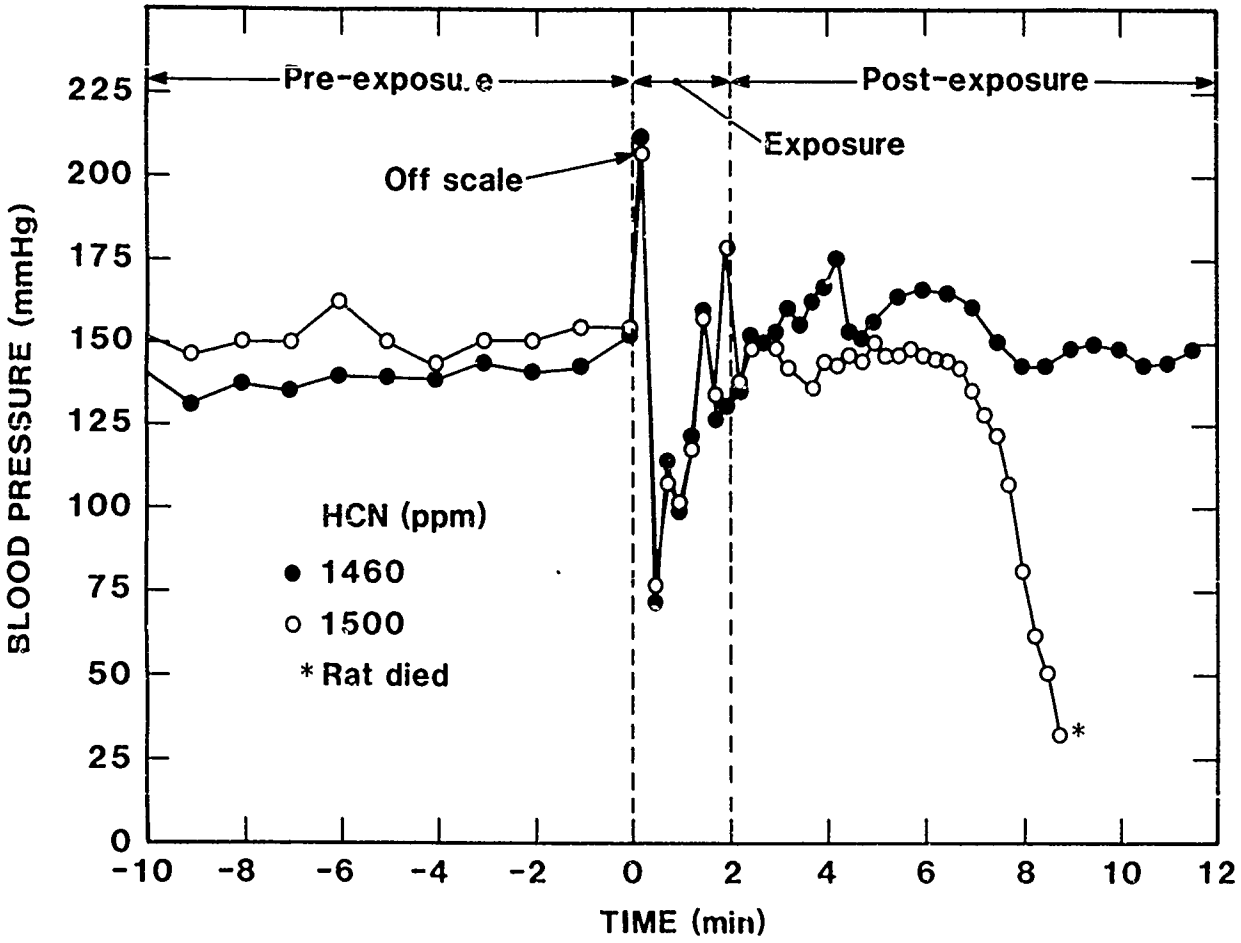
HCN LC_{50} VALUES FOR DIFFERENT EXPOSURE TIMES PLUS 24 HOURS



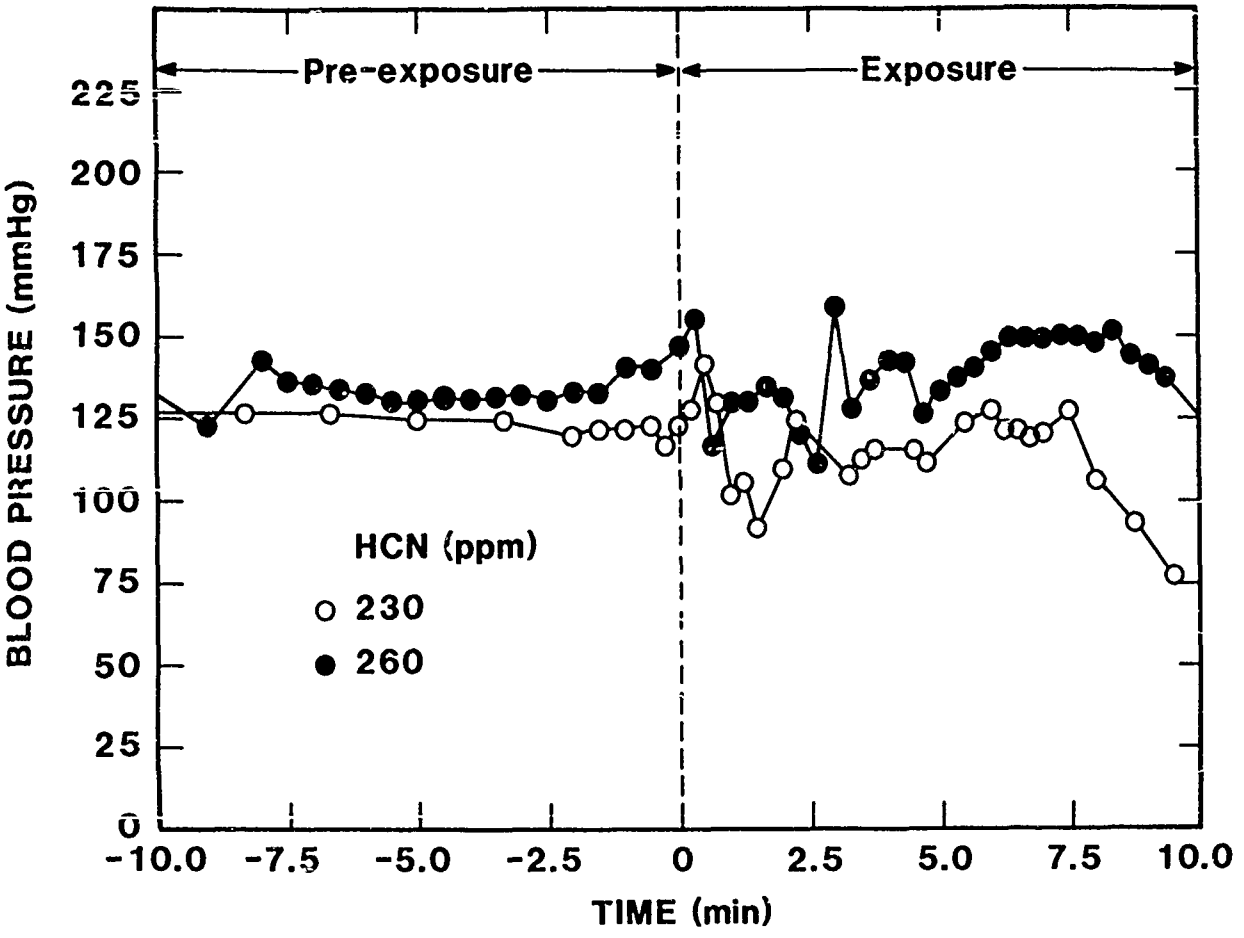
INDIVIDUAL ANIMAL WEIGHTS
FOLLOWING A TEN MINUTE EXPOSURE TO 250 ppm HCN



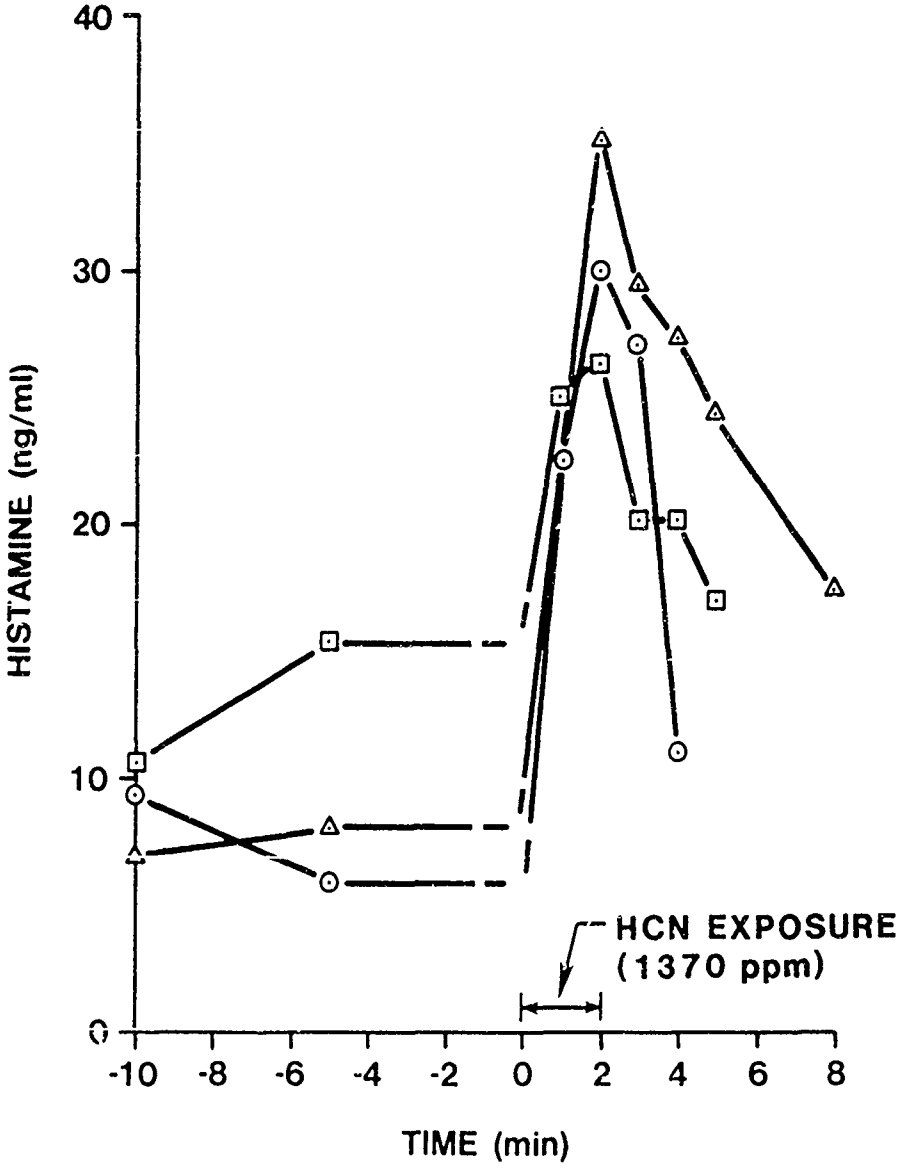
SYSTOLIC BLOOD PRESSURE
TWO MINUTE HCN EXPOSURES



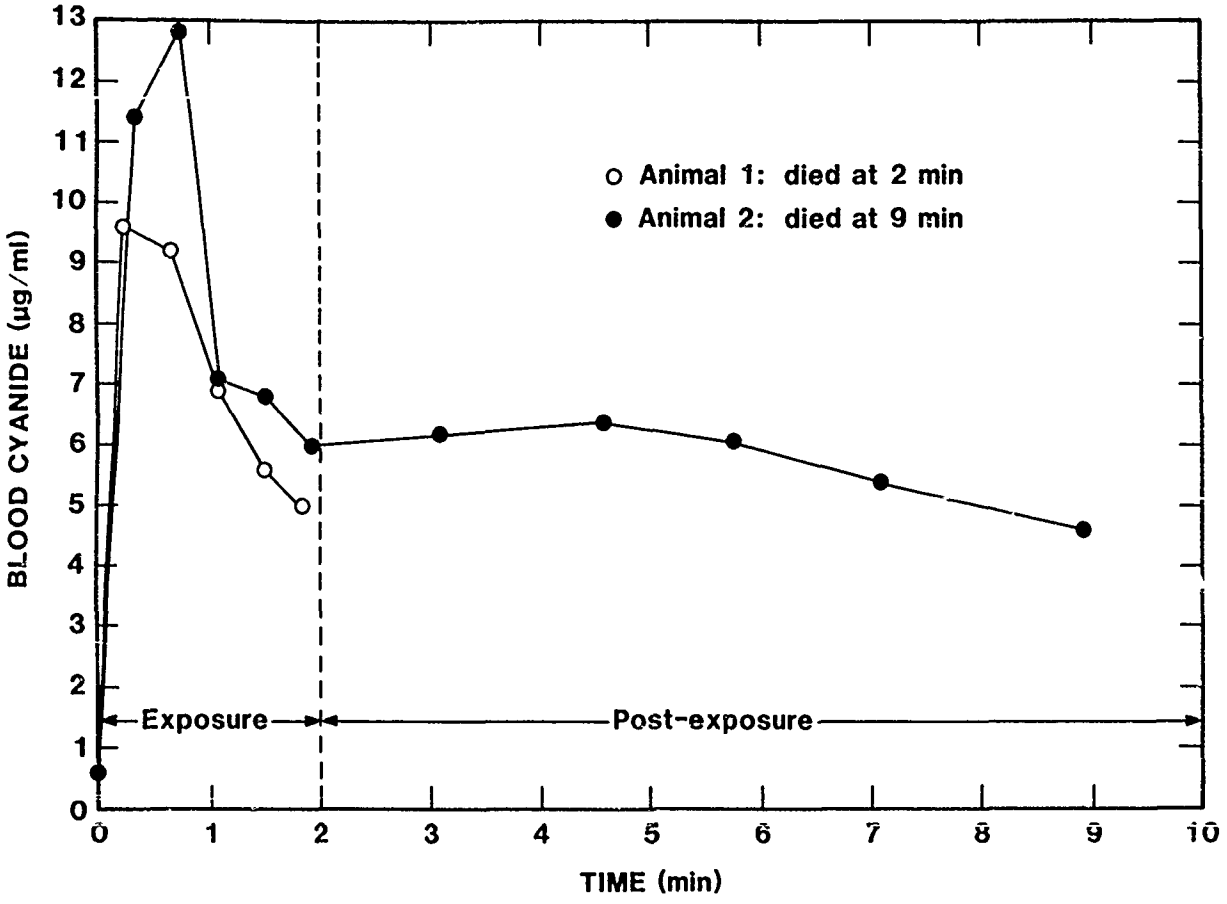
SYSTOLIC BLOOD PRESSURE
TEN MINUTE HCN EXPOSURES



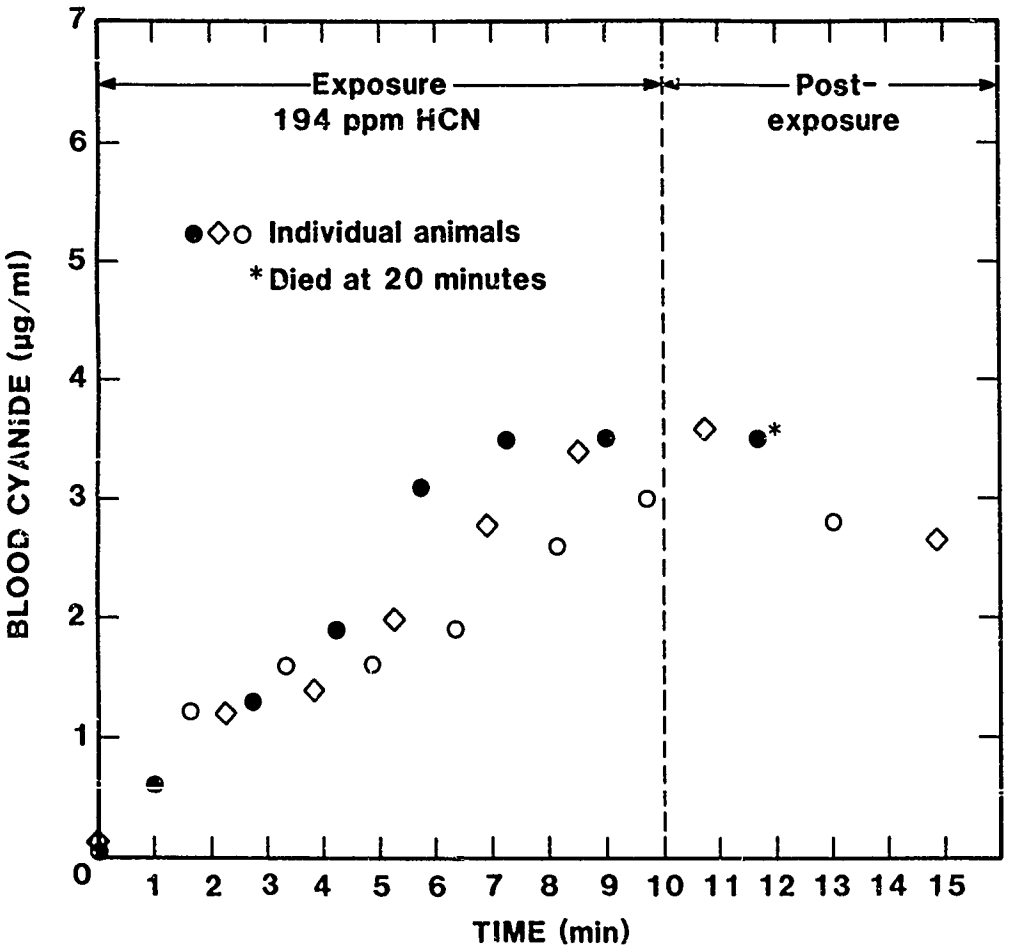
CYANIDE-INDUCED HISTAMINE RELEASE (RAT PLASMA)



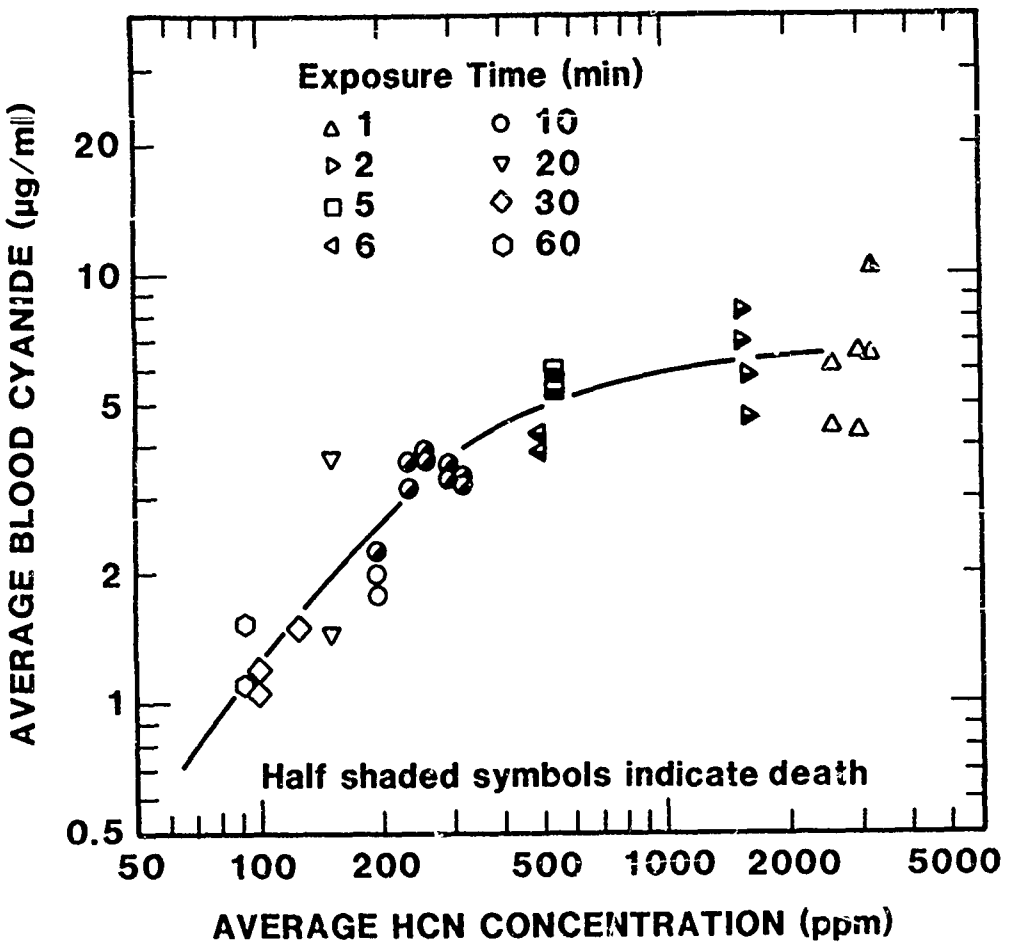
BLOOD CYANIDE LEVELS IN RATS EXPOSED TO 1580 PPM HCN FOR 2 MINUTES



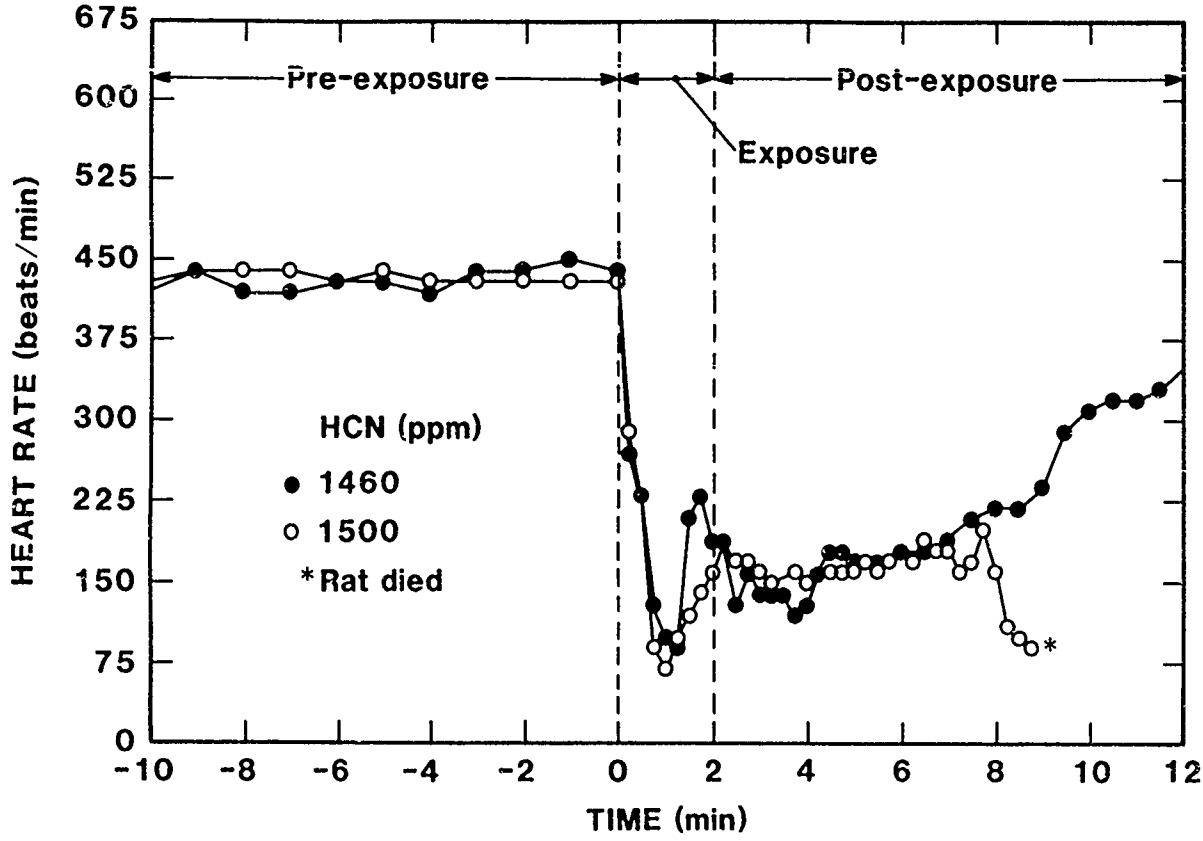
BLOOD CYANIDE MEASUREMENTS DURING 10 MINUTE EXPOSURES TO HCN



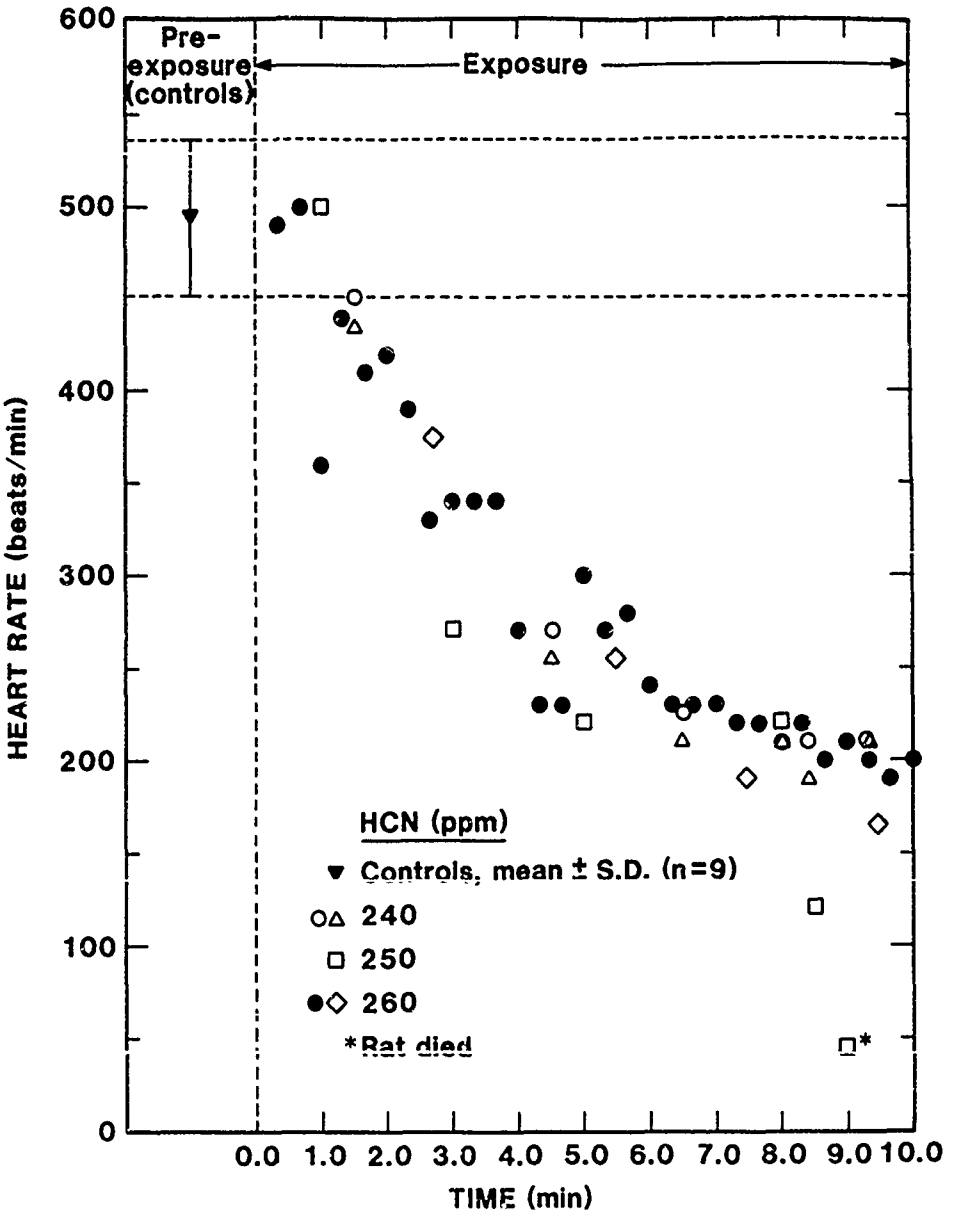
TIME-WEIGHTED AVERAGE BLOOD CYANIDE LEVELS IN ANIMALS EXPOSED TO LC_{50} VALUES OF HCN



HEART RATE RESULTS
TWO MINUTE HCN EXPOSURES



HEART RATE RESULTS
TEN MINUTE HCN EXPOSURES



CONCLUSIONS

A. LETHALITY

- Acute inhalation lethal exposures of HCN ranged from 3000 ppm for 1 minute to 86 ppm for 60 minutes
- These data, with the exception of the 60 minute value, fit the following linear relationship:

$$\ln[\text{time}_{min}] = 8.16 - 1.02 \ln[\text{HCN}_{ppm}]$$

- Most deaths occurred with 24 hours, but some animals died during the post-exposure observation period after losing extensive weight

B. INCAPACITATION

- The incapacitating levels were approximately 65% of the lethal concentrations
- Many incapacitated animals exhibited convulsive seizures during the recovery period

C. BLOOD PRESSURE

- Upon HCN exposure, blood pressure first increased, then decreased, and then slowly returned to normal. Imminent death was indicated by an additional substantial drop in pressure.

D. HEART RATE

- Heart rate decreased in all exposures. The mean decrease ranged from 66-84%.

E. BLOOD CYANIDE

- Time-weighted average blood cyanide levels increased with increasing average atmospheric HCN concentrations
- In most cases, blood cyanide levels increased gradually during exposure and decreased post-exposure
- In a few cases, concentrations of HCN greater than 1500 ppm rapidly produced (15-45 s) a momentarily high peak of blood cyanide (8.6-14.8 µg/ml)
- Animals with time-weighted average blood cyanide levels above 2.0 µg/ml died except some one minute exposures with levels between 4 and 6.5 µg/ml

F. ARTERIAL PLASMA HISTAMINE

- Animals exposed to 2 min LC_{50} levels of HCN showed an immediate increase of plasma histamine with peak levels of 35 ng/ml at 2 minutes. These levels decreased at a slower rate following exposure.

PHYSIOCHEMICAL RESPONSES TO CYANIDE (CN^-) AND THE ANTIDOTAL EFFECTS OF HYDROXOCOBALAMIN IN AN ANIMAL MODEL

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Division of Medicine
Walter Reed Army Institute of Research, Washington, DC 20307-5100

The use of cyanide gas (HCN) by a military adversary in the event of tactical warfare is considered to be a serious possibility. The feasibility of treating troops poisoned by this substance in a combat zone would probably be difficult considering the rapidity with which toxicity occurs and the problems of transporting them to an appropriate treatment facility. Therefore, the development of prophylactic measures that can be used when exposures are likely to occur is of considerable military importance.

Hydroxocobalamin (HO-Cbl) avidly binds cyanide anion (CN^-) to form Vitamin B_{12} (CN-Cbl) which is rapidly excreted via the kidneys if plasma levels of CN-Cbl exceed the plasma protein binding capacity for cobalamins (Cbls). It has been known for some time that HO-Cbl might be a useful antidote against CN^- poisoning but rigorous pharmacologic studies of its use for this purpose have not been done. Pharmacokinetic studies in our laboratory have shown that i.v. administered HO-Cbl clears the kidneys less rapidly than CN-Cbl and thus may provide a reservoir of reactant with which to bind CN^-.

Studies of sublethal CN^- toxicity were conducted in female dogs for which the pharmacokinetics of high dose HO-Cbl and CN-Cbl had been defined. Patterns of cardiorespiratory responses were defined and monitored for calculating doses of i.v. CN^- to be administered. Blood and urine Cbls as well as free and total CN^- were measured.

The administration of NaCN caused reproducible tachycardia followed by bradycardia in the animals. This substance also caused the animals to hyperventilate and continued administration eventually caused apnea. In addition, CN^- caused an initial rise in the blood pressure followed by hypotension with continued administration.

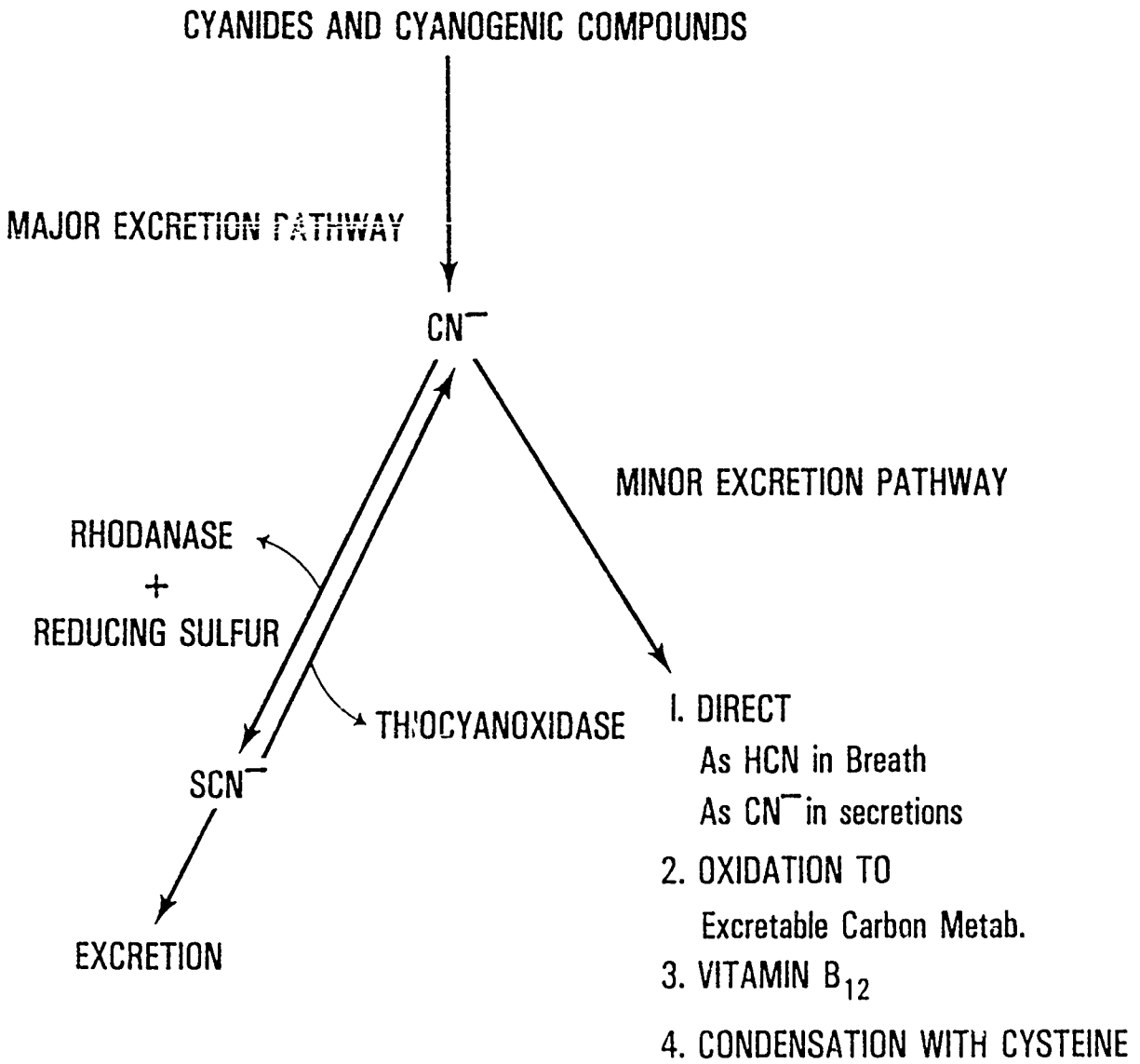
Dogs given bolus injections of NaCN received an average cumulative dose of 1.59 mg/kg before hypotension occurred. In dogs pretreated with HO-Cbl before bolus NaCN administration, an average cumulative dose of 2.48 mg/kg was required to produce comparable toxicity. Dogs given NaCN by continuous infusion received even higher doses before equivalent hypotension was observed.

Additional studies indicated that the reaction between HO-Cbl and CN^- proceeds rapidly and that the strength of binding between CN^- and Cbl increases with time. Chromatographic analyses of blood and urine specimens from animals given sublethal doses of CN^- in the presence of HO-Cbl confirmed the *in vivo* binding of CN^- by Cbl.

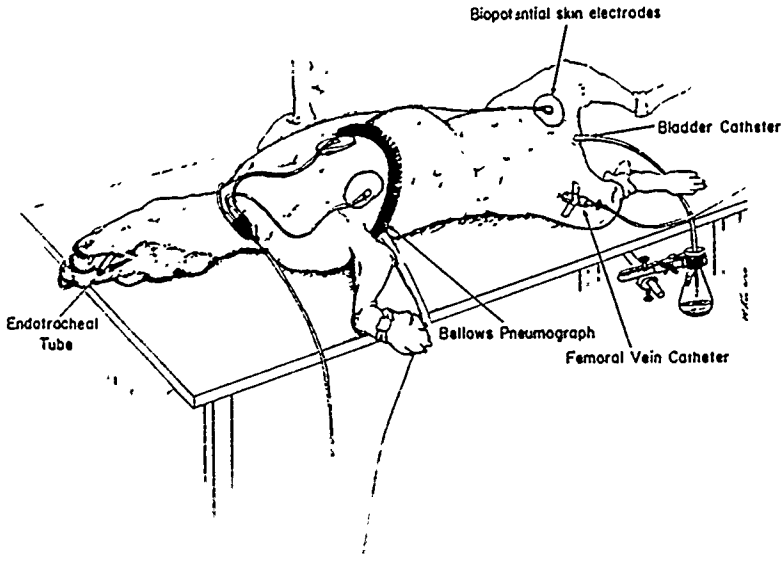
In summary, our studies indicate that HO-Cbl loading increases the resistance of experimental animals to the cardiovascular and respiratory toxicity of cyanide by providing a vehicle for binding and renal excretion of CN^-. Definition of the potential usefulness of these antidotal effects of HO-Cbl awaits further study.

PURPOSE

To determine the efficacy of using hydroxocobalamin (HO-Cbl) as an antidote for cyanide (CN^-) poisoning.



Body excretion pathways of cyanides and cyanogenic compounds



EXPERIMENTAL PROCEDURES

1. Immediately after sedation, the following catheters were placed:

   a. Indwelling catheter in the femoral artery for monitoring blood pressure and heartrate.

   b. Indwelling catheter in the femoral vein for administering i.v. fluids.

   c. Indwelling catheter in the external jugular vein for collecting blood samples.

   d. Foley catheter in the bladder for urine collection.

2. Respiration was measured using a Bellows Respiratory Transducer.

3. Baseline blood and urine samples were collected for cyanide and HO-Cbl measurements.

4. Ringer's Lactate infusion was started and maintained at 1.5 ml/min to establish stable urine flow.

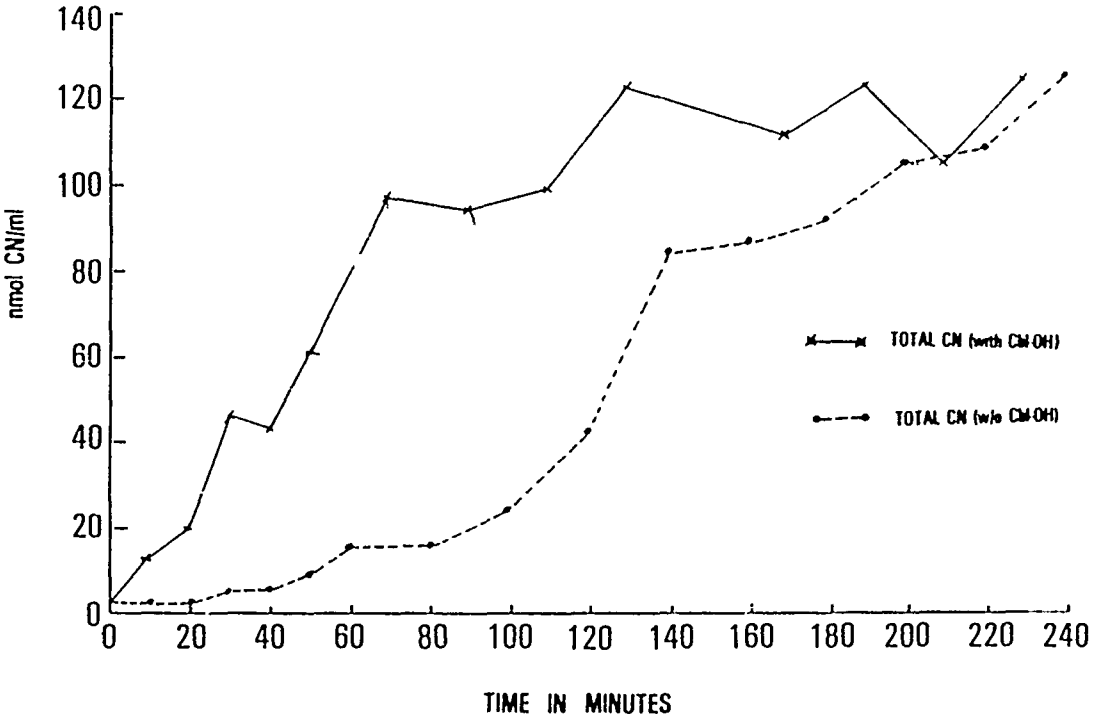
5. Hydroxocobalamin (HO-Cbl) infusion started where designated.

6. Cyanide administered, either as bolus injection time intervals.

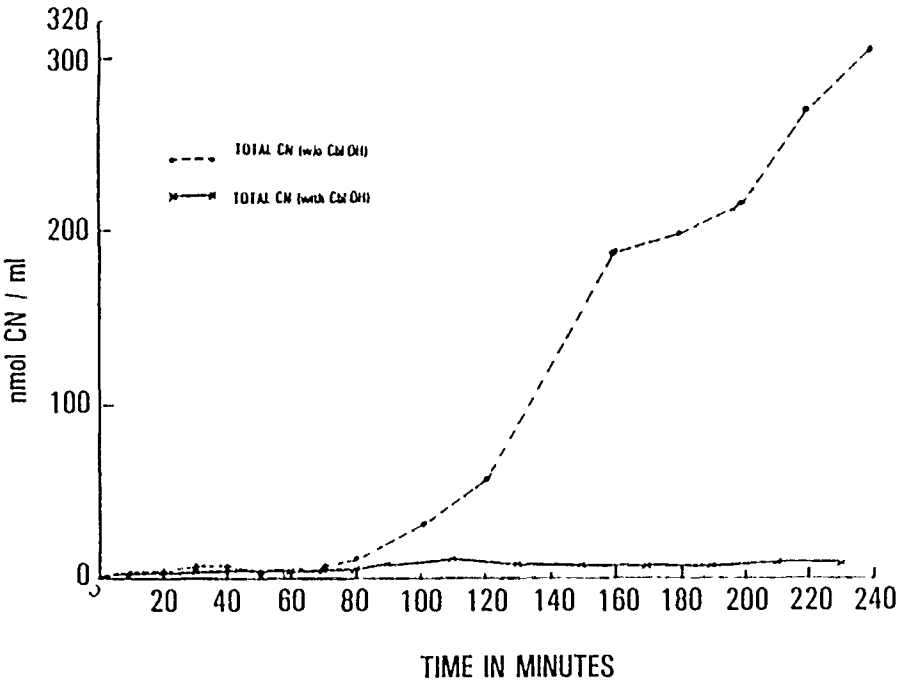
7. Blood and urine were sampled at designated time intervals.

8. Cyanide was measured in blood and urine by the method of D. Johnson.

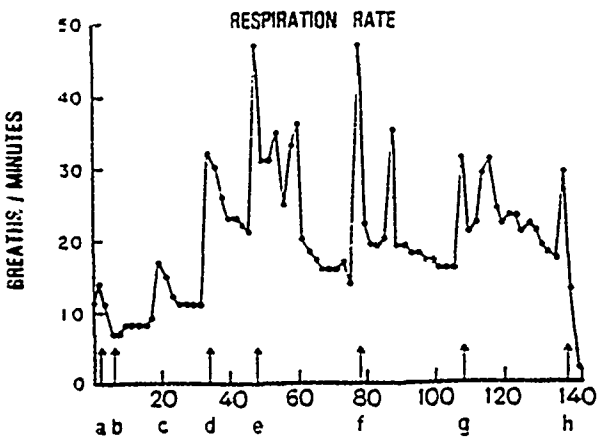
9. HO-Cbl content in plasma and urine were measured using modified methods of V. Herbert and R. Green, respectively, and GFR's were measured after the method of H.W. Smith.

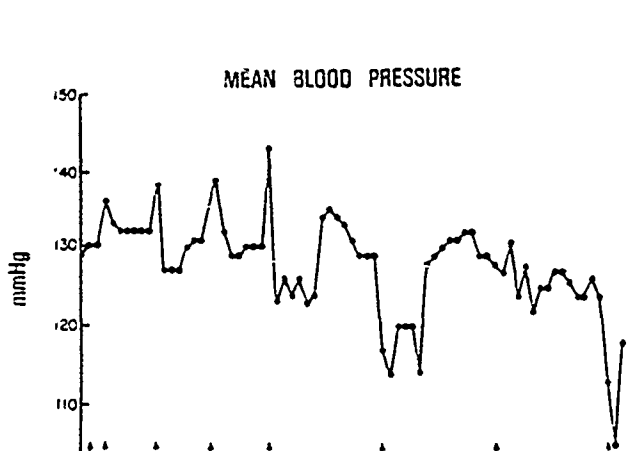


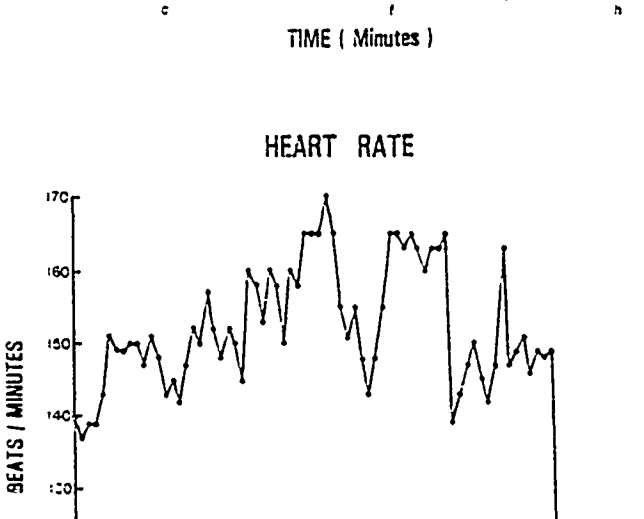
COMPARISON OF BLOOD CYANIDE VALUES OF DOGS GIVEN CONSTANT INFUSION OF NaCN AT 0.02mg/Kg/min ; ONE WITH Cbl–OH INFUSION, THE OTHER WITHOUT
(FLOW RATE = 1ml/min)



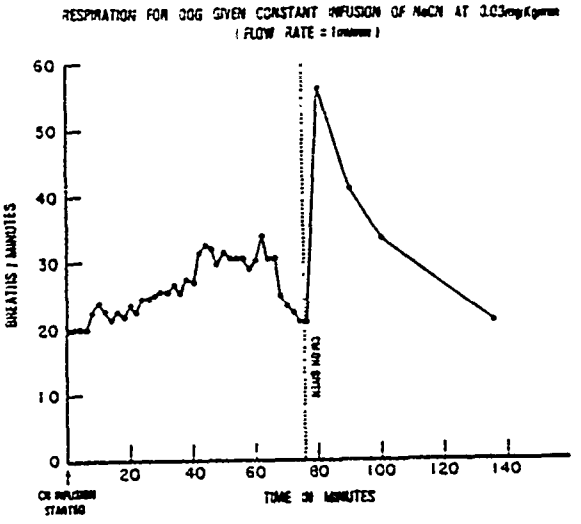
COMPARISON OF URINE CYANIDE VALUES OF DOGS GIVEN CONSTANT INFUSION OF NaCN AT 0.02mg/Kg/min , ONE WITH Cbl–OH INFUSION, THE OTHER WITHOUT
(FLOW RATE = 1ml/min)

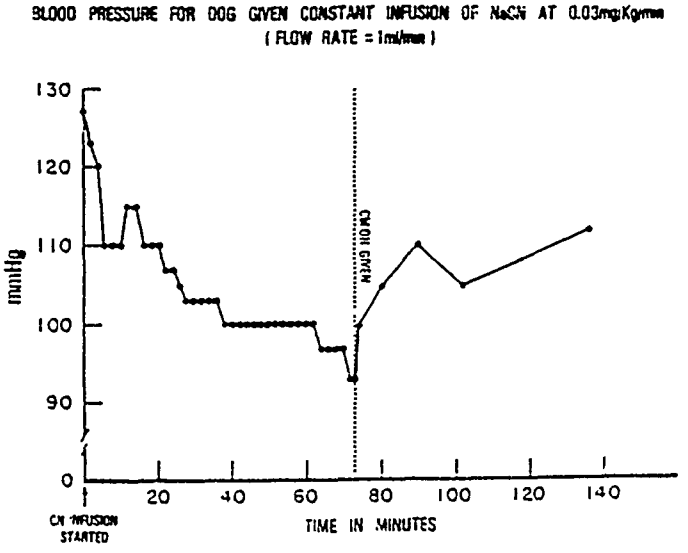


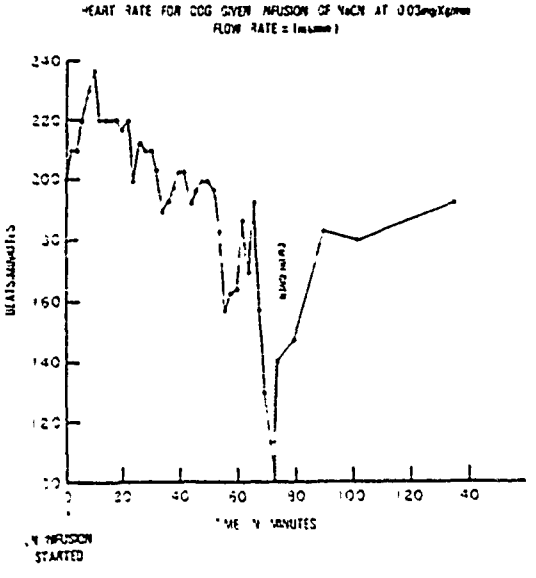




Physiological effects on 5 dogs receiving cumulative doses of NaCN. All 5 received a minimum cumulative amount of 1.0 mg/kg. Of these 5, 1 maximized at 1.55 mg/kg and 2 at 2.15 mg/kg.

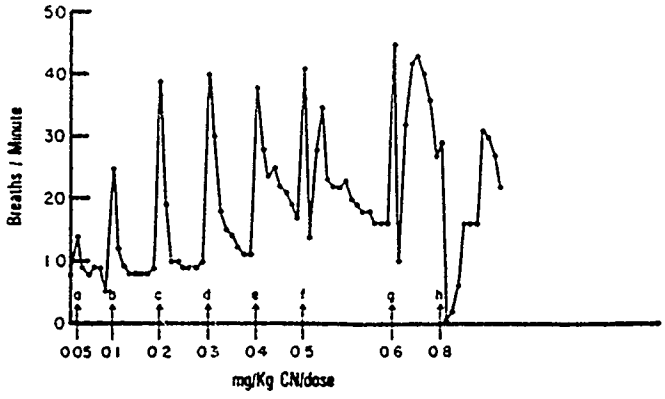




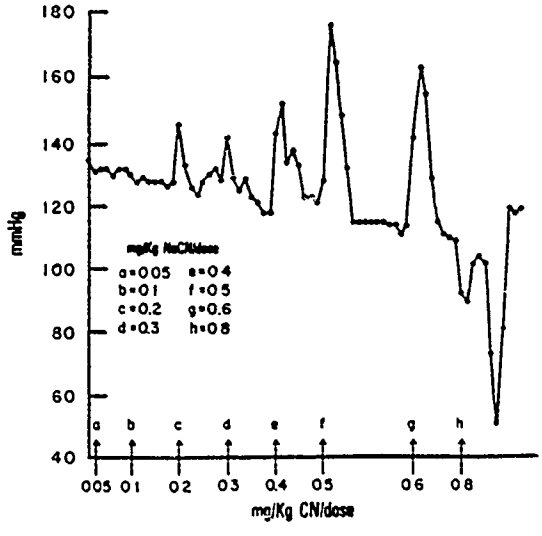


Shown above are the responses of a dog given 0.03 mg/kg/min of NaCN followed by a rescue dose of HO-Cbl at the point indicated. Notice that after the administration of HO-Cbl, the responses approach normalcy.

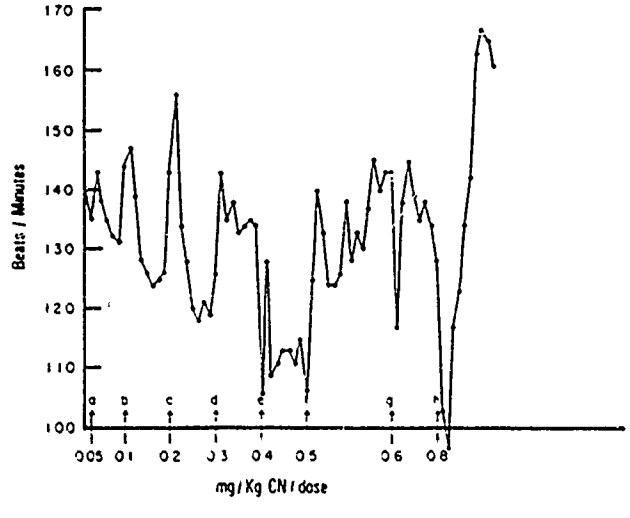
RESPIRATION RATE FOR DOGS GIVEN BOLUS DOSES OF CYANIDE AND INFUSED VITAMIN B_{12a}



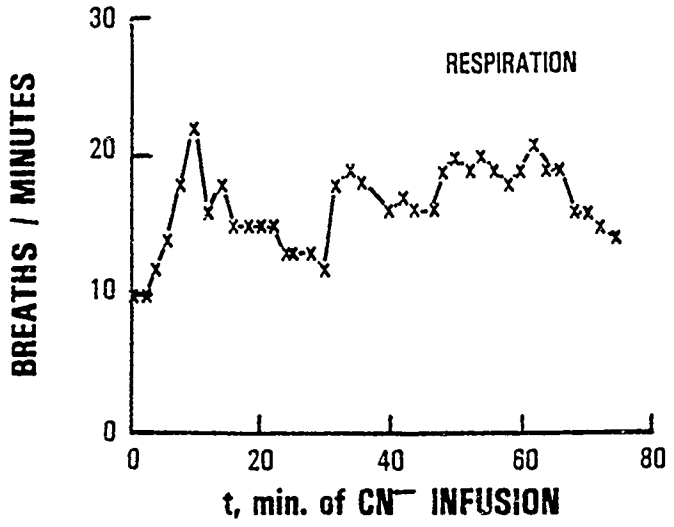
BLOOD PRESSURE FOR DOGS GIVEN BOLUS DOSES OF CYANIDE AND INFUSED VITAMIN B_{12a}

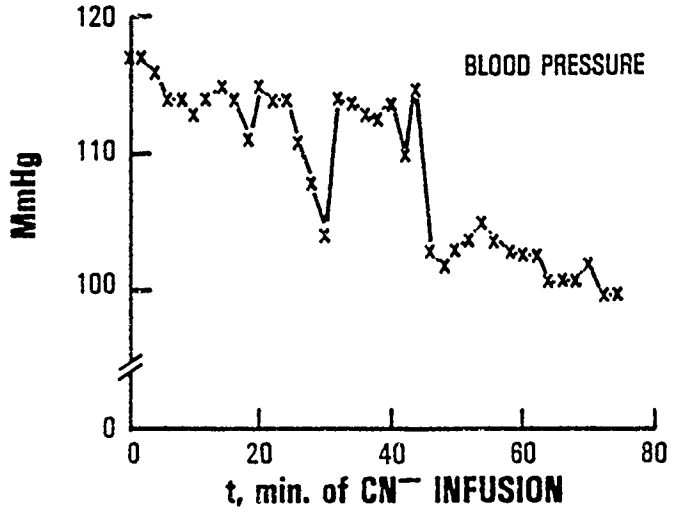


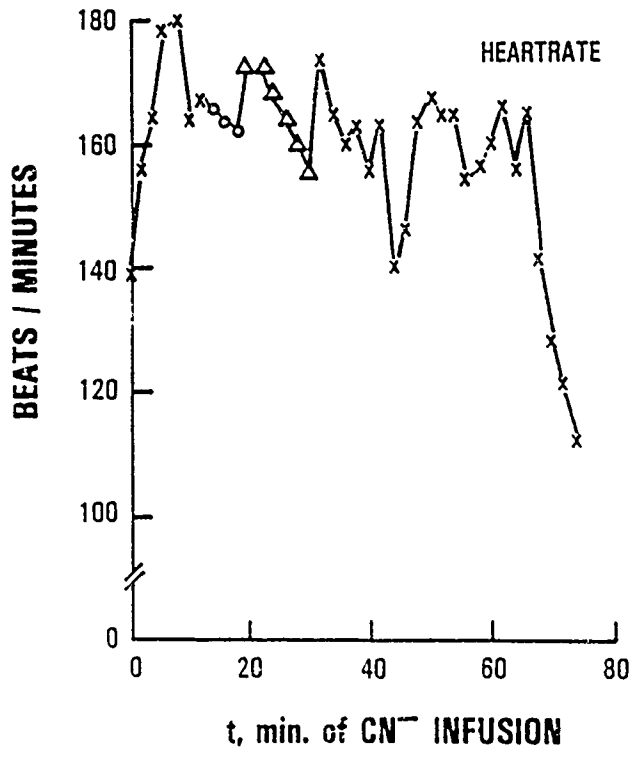
HEART RATE FOR DOGS GIVEN BOLUS DOSES OF CYANIDE AND INFUSED VITAMIN B_{12a}



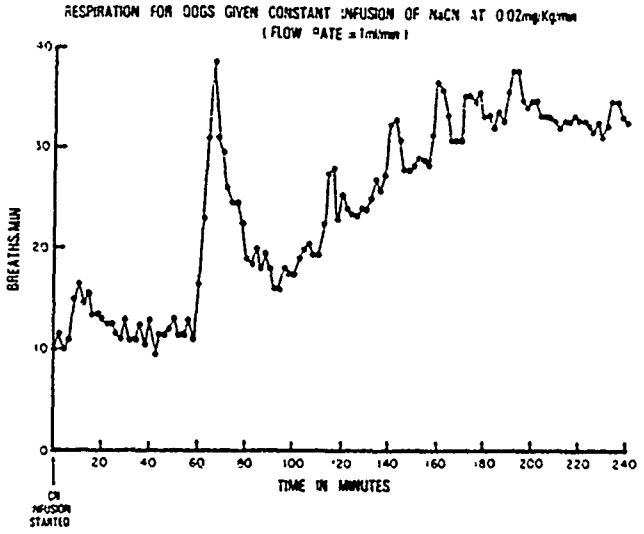
Physiological responses of 3 dogs receiving cumulative doses of NaCN while being continuously infused with HO-Cbl at a rate to maintain plasma level at 0.02 mg/ml. The minimum NaCN dose received was equivalent to 1.55 mg/kg. Of these 3 dogs, 2 received maximum equivalents of 2.95 mg/kg.

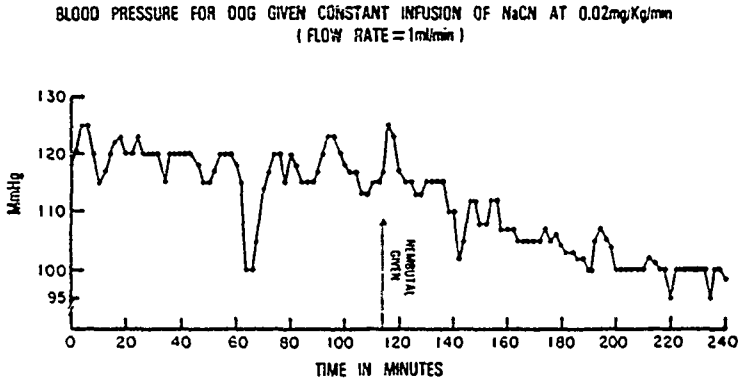


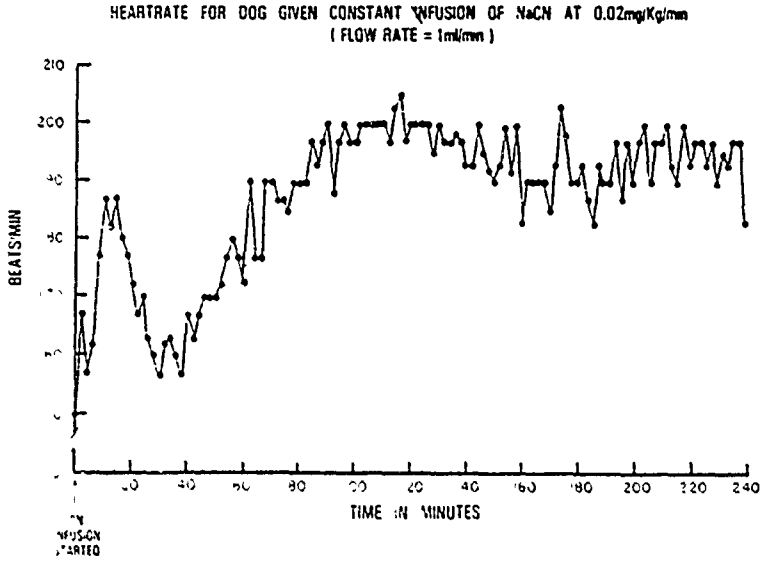




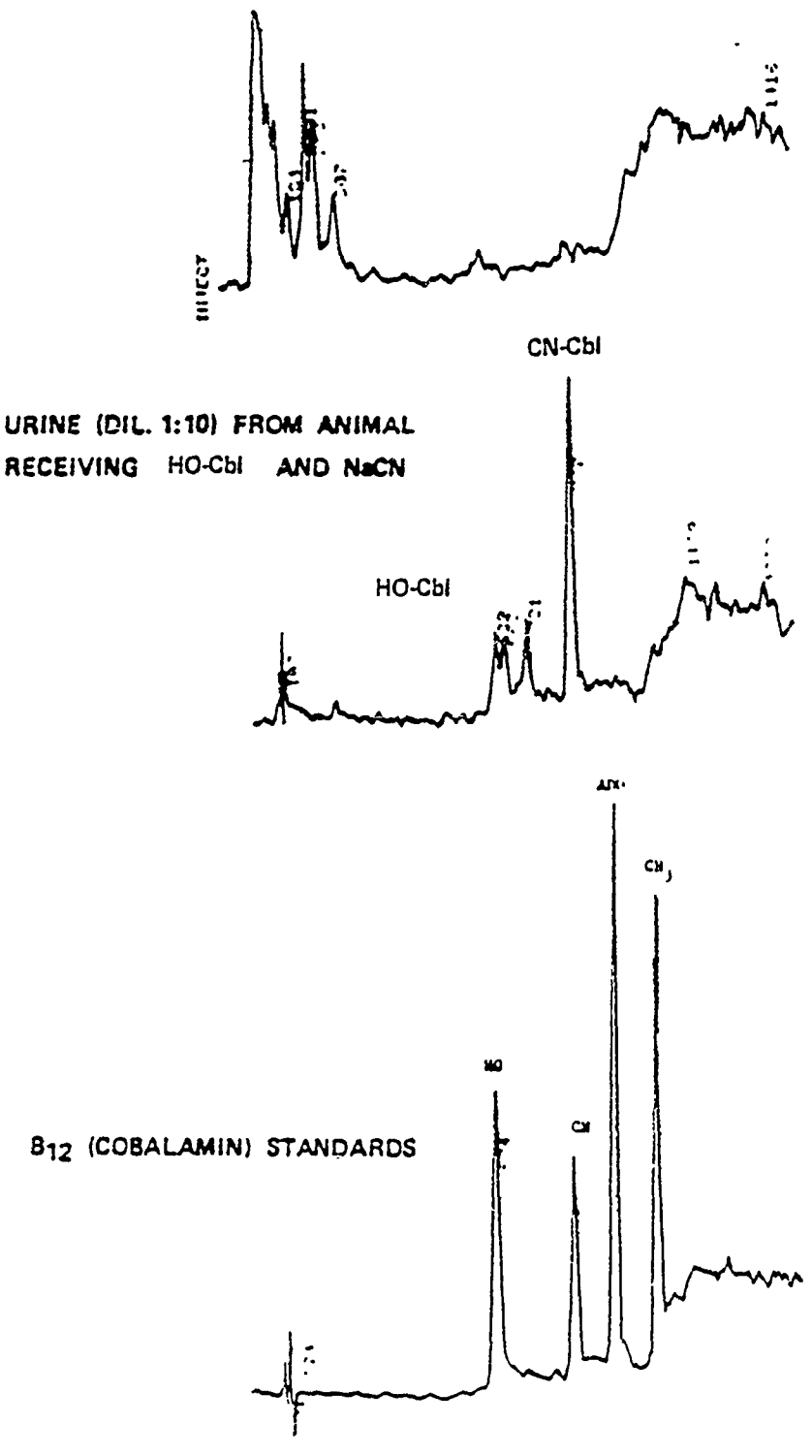
Physiological responses of 6 dogs given NaCN i.v. at a rate of 0.03 mg/kg/min.



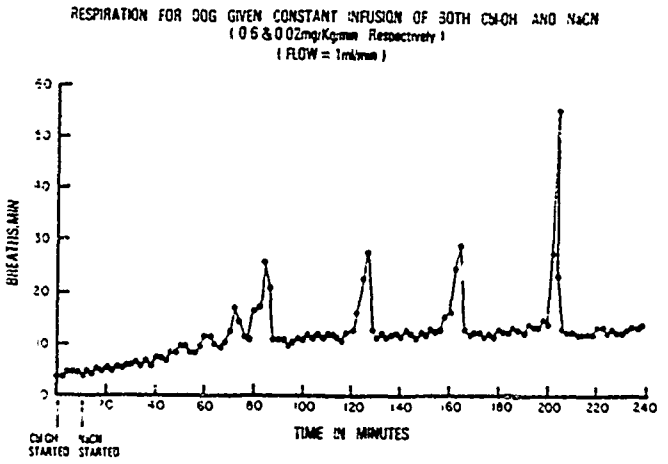


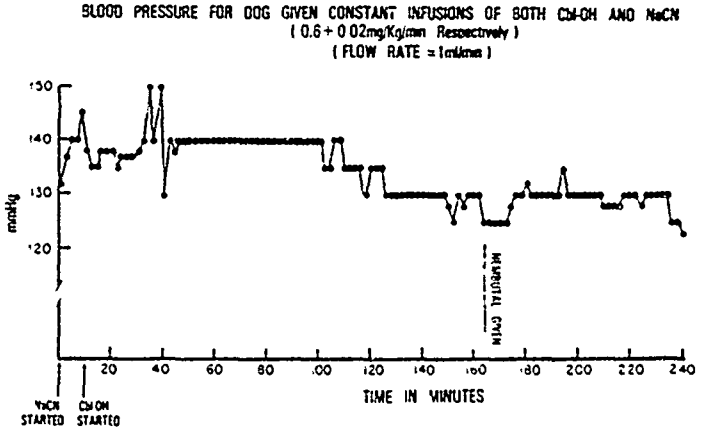


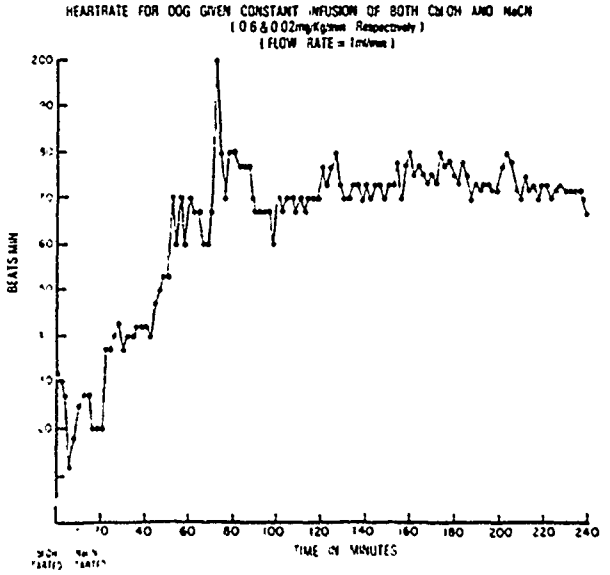
Measurement of respiration, blood pressure and heart rate in dog given an i.v. dose of NaCN at a rate of 0.02 mg/kg/min.



HPLC separation of cobalamins (Vitamin B_{12}'s). Shown at the top is a chromatogram of urine before the appearance of cobalamin. The middle curve shows the partial reaction between HO-Cbl and NaCN. Indicated are the peaks for unreacted HO-Cbl and CN-Cbl formed. The unlabeled peak is an intermediate anionic form. The bottom figure is a chromatogram of Cbl standards of 0.05 mg/ml where HO=hydroxycobalamin, CN=cyanocobalamin, Ado=adenosylcobalamin and CH_3=methylcobalamin. Instrument sensitivity was 0.04 a.u.f.







Measurement of respiration, blood pressure and heart rate in dog given an i.v. dose of NaCN at a rate of 0.02 mg/kg/min while simultaneously being infused with HO-Cbl at a rate of 0.6 mg/kg/min.

EFFECTS OF HO-Cbl LOADING

	EFFECTIVE CUMULATIVE BOLUS DOSES OF CYANIDE	
	CONTROL	IN THE PRESENCE OF HO-Cbl
Respiration Rate Does Not Return to Baseline by 15 min.	0.35 mg/Kg	0.65 mg/Kg
Initial Rise in Blood Pressure	0.05 mg/Kg	0.20 mg/Kg
Fall in Blood Pressure	1.05 mg/Kg	2.95 mg/Kg

CONCLUSIONS

1. The administration of NaCN caused reproducible tachycardia followed by bradycardia in the animals. This substance also caused the animals to hyperventilate and continued administration eventually caused apnea. In addition, CN^- caused an initial rise in the blood pressure followed by hypotension with continued administration.
2. Dogs given bolus injections of NaCN received an average cumulative dose of 1.59 mg/kg before hypotension occurred. In dogs pretreated with HO-Cbl before bolus NaCN administration, an average cumulative dose of 2.48 mg/kg was required to produce comparable toxicity. Dogs given NaCN by continuous infusion received even higher doses before equivalent hypotension was observed.
3. Additional studies indicated that the reaction between HO-Cbl and CN^- proceeds rapidly and that the strength of binding between CN^- and Cbl increases with time. Chromatographic analyses of blood and urine specimens from animals given sublethal doses of CN^- in the presence of HO-Cbl confirmed the *in vivo* binding of CN^- by Cbl.

REFERENCES

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EFFECTS OF NERVE AGENTS ON THE RESPIRATORY AND CARDIOVASCULAR SYSTEMS

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1. INTRODUCTION and METHODS

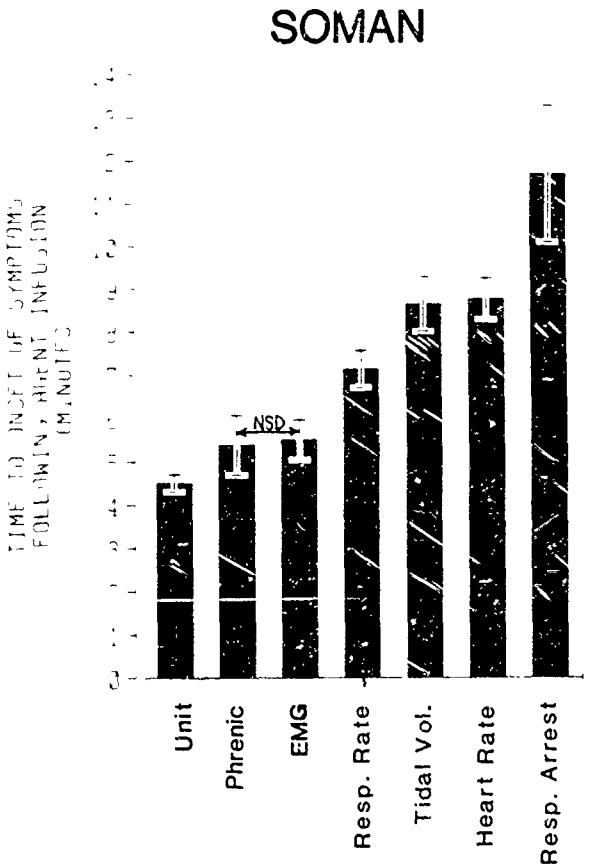
Soman (GD), sarin (GB), tabun (GA), and VX are organophosphate cholinesterase inhibitors. Disagreement exists concerning both the sites and mechanisms of actions thought to be responsible for these agents' toxicity. This study examined the relative toxicity of these agents' acute actions on the central nervous system, neuromuscular junction, and cardiovascular system.

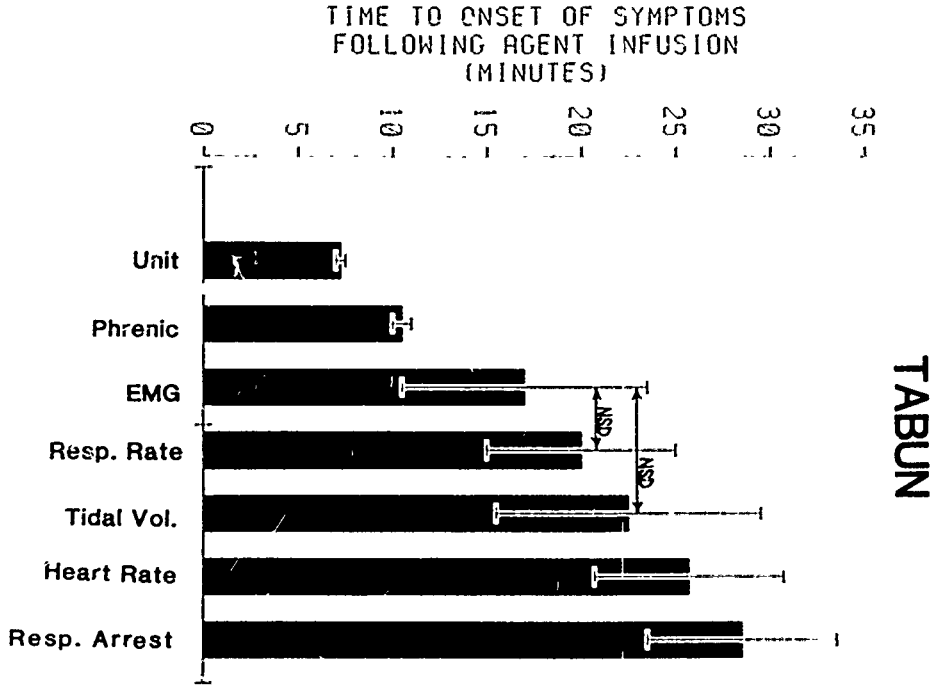
Using spontaneously breathing cats anesthetized with DIAL (70 mg/kg), recordings were made of: i) medullary respiratory-related unit activity (Unit), ii) phrenic nerve activity (Phr), iii) diaphragm electromyographic activity (EMG), iv) contractions of the diaphragm leaflet, v) airflow, vi) femoral arterial pressure (AP), and vii) electrocardiographic activity (ECG), as well as monitoring blood gases and expired CO2. The agents were infused at the rate of 1 LD50 per 15 minutes until cessation of spontaneous respiration, at which time the phrenic nerve was stimulated supramaximally to test diaphragmatic contraction. The nerve was stimulated with 2 msec pulses for 0.5 sec at 10 Hz and at 100 Hz. Artificial ventilation was initiated and agent infusion was then resumed at 3 times the initial rate, while diaphragm contraction was tested periodically.

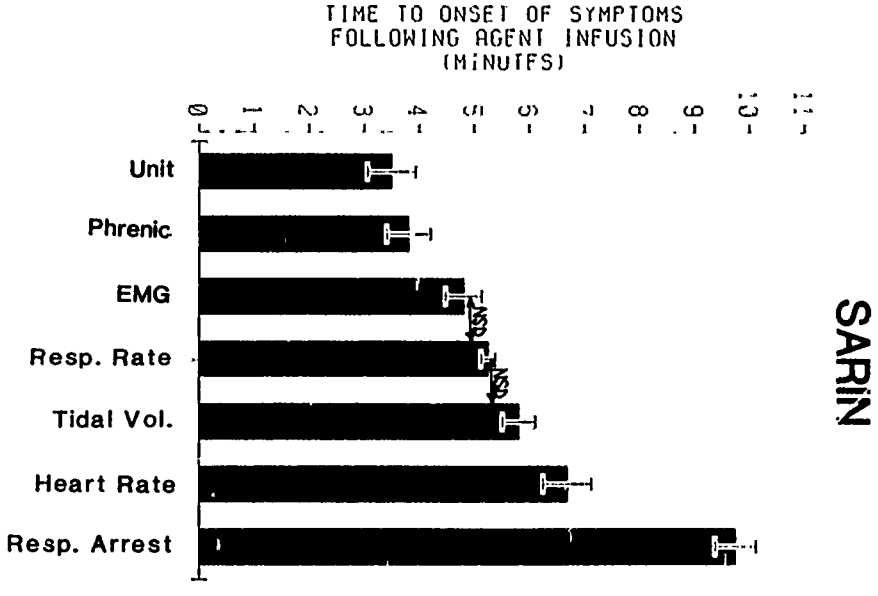
2. RESULTS

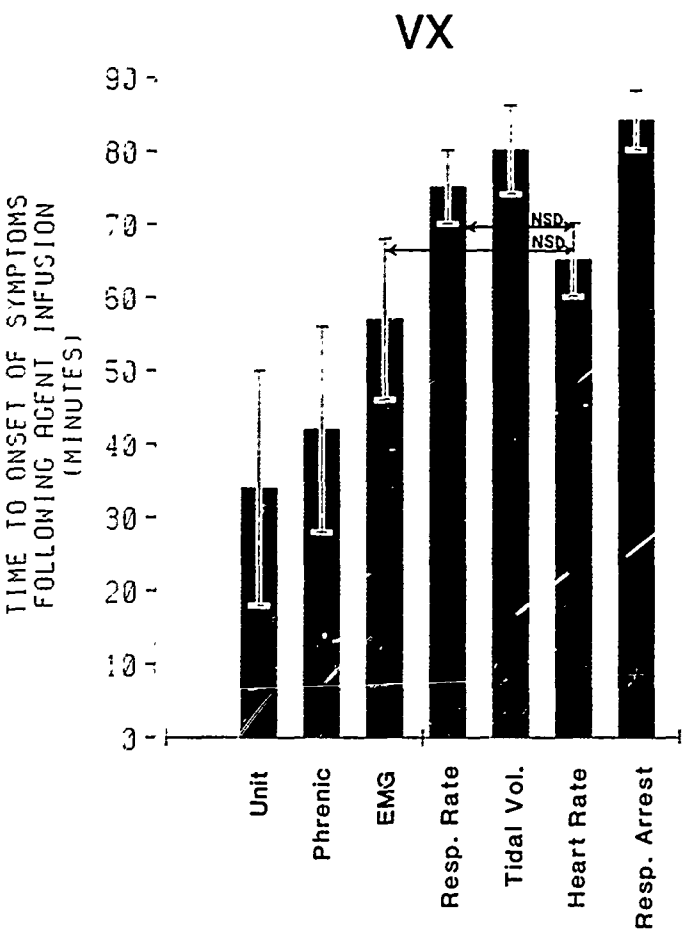
Sensitivity of Selected Variables - Time to first sign of change in the variable following agent infusion

All variables are significantly different from all others ($p < 0.05$) except where indicated as not significantly different (NSD).

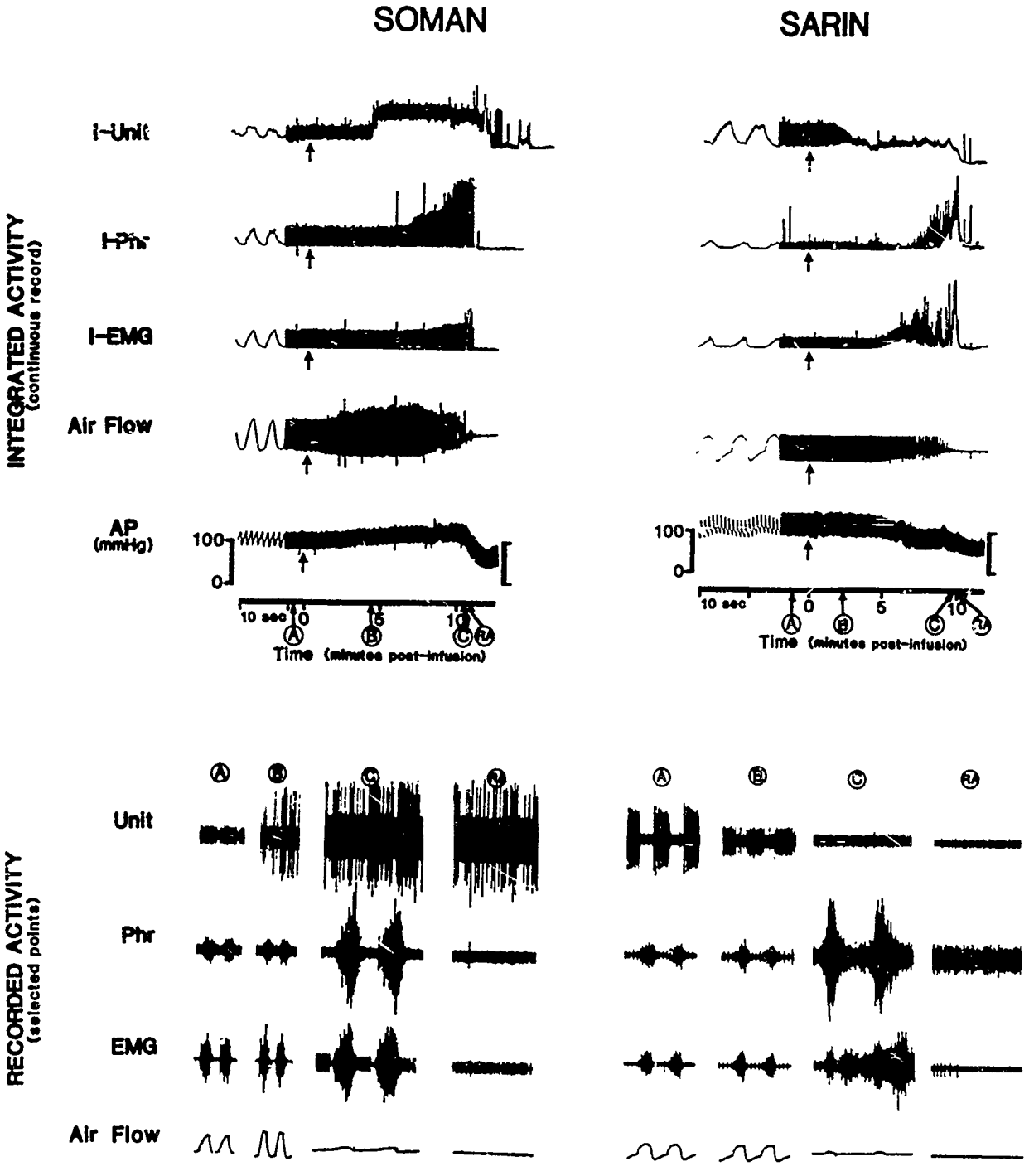



TIME TO ONSET OF SYMPTOMS
FOLLOWING AGENT INFUSION
(MINUTES)
0
5
10
15
20
25
30
35
Unit
Phrenic
EMG
Resp. Rate
Tidal Vol.
Heart Rate
Resp. Arrest
NSD
NSD
TABUN


TIME TO ONSET OF SYMPTOMS
FOLLOWING AGENT INFUSION
(MINUTES)
0
1
2
3
4
5
6
7
8
9
10
11
Unit
Phrenic
EMG
Resp. Rate
Tidal Vol.
Heart Rate
Resp. Arrest
NSD
NSD
SARIN


VX
TIME TO ONSET OF SYMPTOMS
FOLLOWING AGENT INFUSION
(MINUTES)
90
80
70
60
50
40
30
20
10
0
NSD
NSD
Unit
Phrenic
EMG
Resp. Rate
Tidal Vol.
Heart Rate
Resp. Arrest

Sequence of the Toxic Syndrome – Typical recordings for soman, sarin, tabun, and VX



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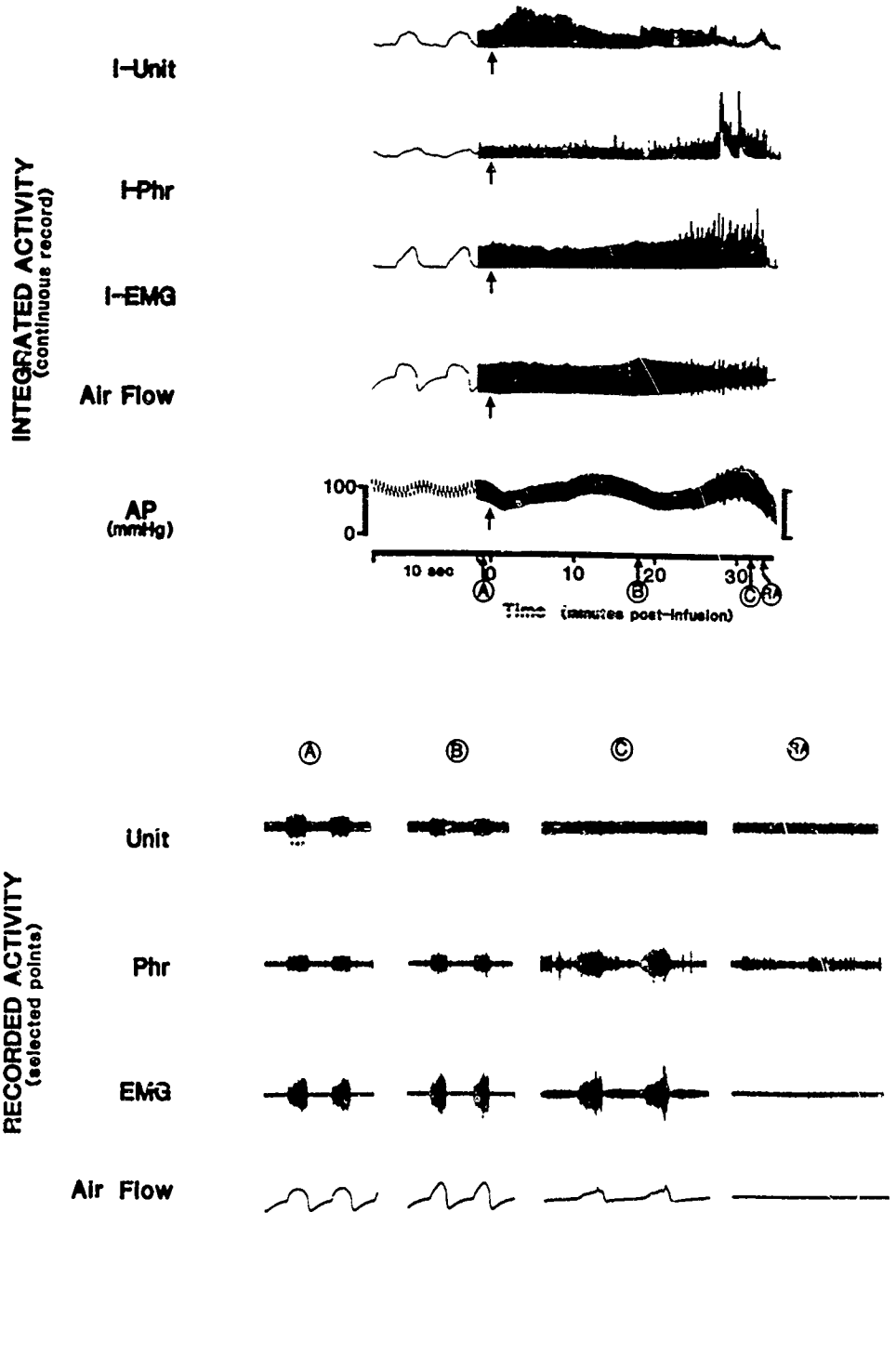
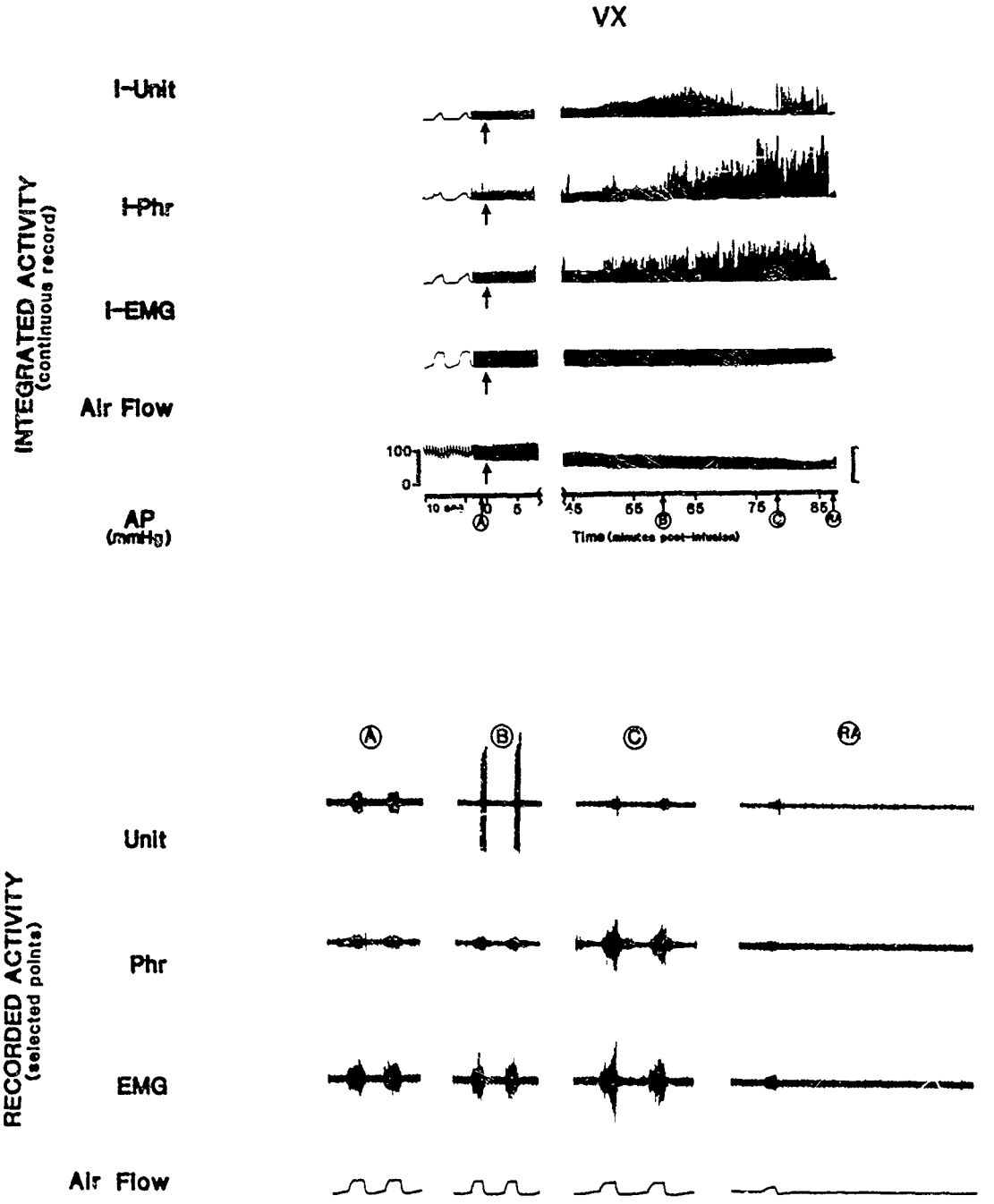

INTEGRATED ACTIVITY
(continuous record)
I-Unit
I-Phr
I-EMG
Air Flow
AP
(mmHg)
100
0
10 sec
0
10
20
30
Time (minutes post-infusion)
A
B
C
RECORDED ACTIVITY
(selected points)
Unit
Phr
EMG
Air Flow


VX
INTEGRATED ACTIVITY
(continuous record)
I-Unit
I-Phr
I-EMG
Air Flow
AP
(mmHg)
100
0
10 sec
0
5
45
55
65
75
85
Time (minutes post-infusion)
RECORDED ACTIVITY
(selected points)
Unit
Phr
EMG
Air Flow

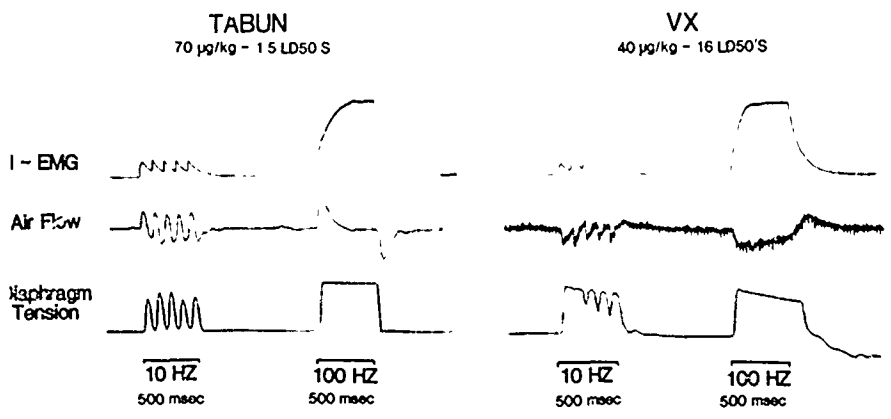
RANK-ORDER OF TOXICITY

The variables are ranked in the order in which they begin to be affected by agent; the rankings are for pooled data, all animals and all agents. All variables are significantly different from all others ($p < 0.05$), except EMG and respiratory rate (RR).

1. Unit
2. Phrenic
3. EMG — NSD
4. Respiratory Rate — NSD
5. Tidal Volume
6. Heart Rate
7. Respiratory Arrest

Phrenic Nerve Stimulation

STIMULATE PHRENIC NERVE AFTER RESPIRATORY ARREST



3. CONCLUSIONS

Of the variables recorded, medullary respiratory-related unit activity is the most sensitive indicator of agent-induced respiratory distress. Unit activity consistently becomes disrupted prior to changes in the other variables. This is followed by changes in phrenic nerve activity, diaphragm EMG, respiratory rate, airflow and heart rate. Just prior to cessation of spontaneous respiration, both phrenic and EMG frequently exhibit increases in activity; often this increase in activity is accompanied by erratic firing and noise spikes. In addition, blood pressure frequently exhibits a biphasic response with an increase in pressure followed by a decrease to below control values just prior to respiratory arrest. Moreover, cessation of spontaneous respiration precedes cardiovascular collapse, even though the animal's cardiovascular system becomes increasingly compromised. The decrease in tidal volume during the animal's last breaths may be attributed to I) increased airway resistance or II) the erratic discharge of the phrenic nerve causing inadequate or ineffective muscular contractions. The diaphragm neuromuscular junction appears to be functional at respiratory arrest as evidenced by the increased EMG and the fact that the diaphragm muscle still contracts tetanically when challenged with a 100 Hz train of 2 msec pulses for 500 msec. At the time of cessation of spontaneous respiration, the respiratory-related unit, phrenic nerve, and EMG all cease firing and the diaphragm neuromuscular junction is functional. Our findings support previous studies in barbiturate-anesthetized animals showing that the central nervous system is most sensitive to nerve agent intoxication and that loss of central respiratory drive is the predominant feature of agent-induced respiratory arrest (de Candole et al., 1953 and Meeter & Wolthius, 1968).

A NON-INVASIVE HUMAN SWEAT GLAND ASSAY FOR QUANTIFYING REACTIVITY TO CHOLINERGIC AGONISTS AND ANTAGONISTS

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INTRODUCTION

The eccrine sweat glands in man are a distributed cholinergic exocrine gland network of relatively high density (100-200 sq cm) over the general body surface. With miniature self-contained electronic evaporative capsules the sweat production on several adjacent skin areas can be accurately measured simultaneously and compared as a function of time. These non-invasive measurements could form the basis for an *in vivo* assay if coupled with a non-invasive method for pharmacologically stimulating sweat secretion with a cholinergic agonist.

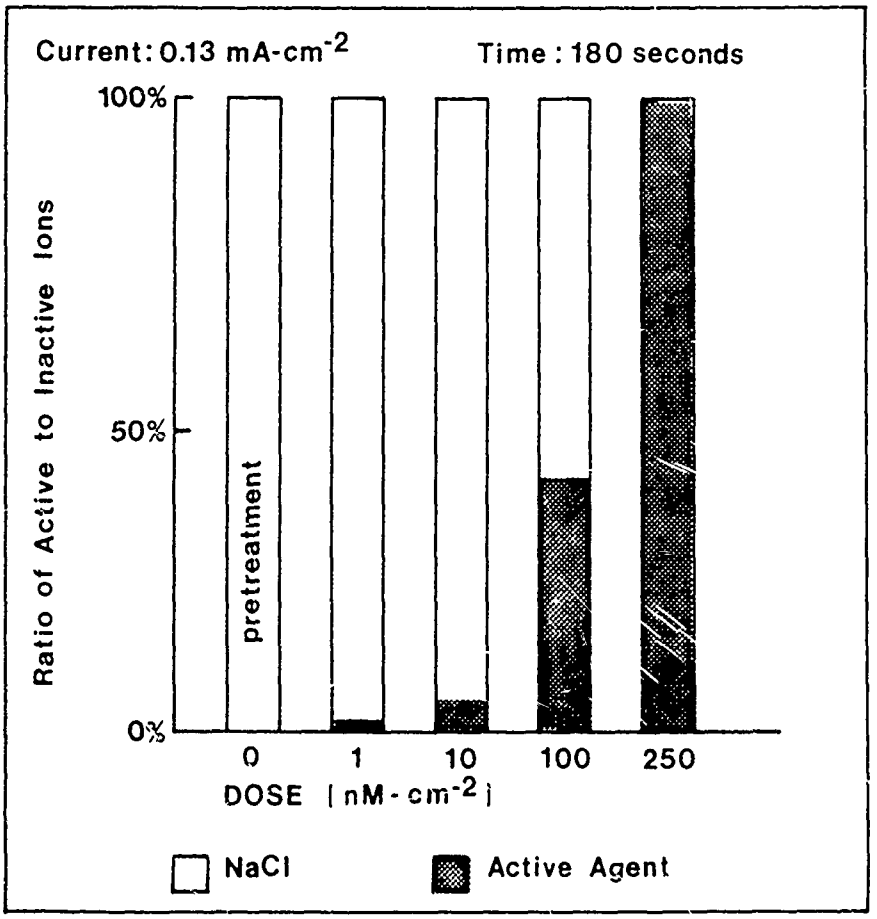
Iontophoresis is an electrophoretic method for safely and painlessly transferring the cationic salts of drug molecules across the skin barrier. In the past, iontophoresis has been a qualitative method with no prescribed procedure for adjusting delivered dose. The purpose of this paper is to describe progress on the development of a safe, non-invasive human assay for cholinergic reactivity and anticholinergic potency and to present results of some studies in which the assay has been applied as a tool

THEORY

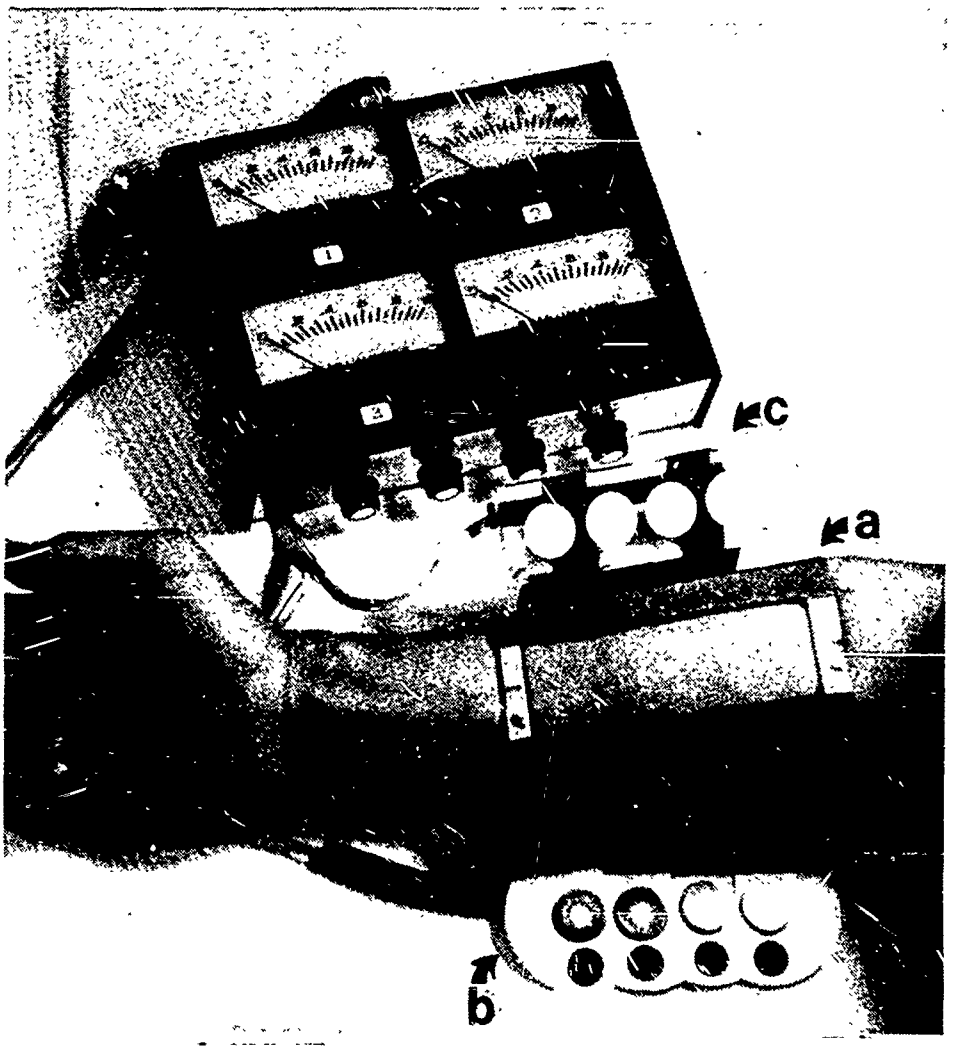
By Faraday's Law the total quantity of cationic material transported into the skin layers is directly related to the product of current and time and inversely related to the valency of the cation.

$$Q = (It)/(zF)$$

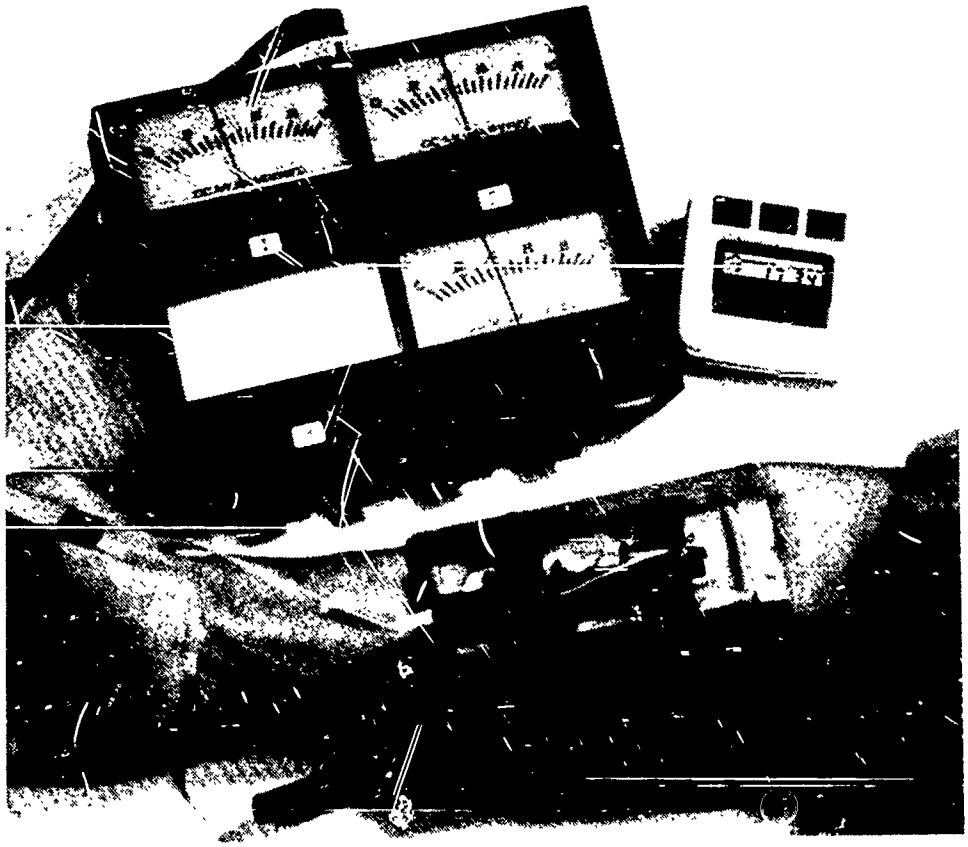
where Q is the molar quantity of cations transported into the skin in nM/sq cm, I is the current density in mA/sq cm, t is the duration of iontophoresis in sec, z is valence of the cation, and F is Faraday's constant (.0965 mA-sec/nM). In the present studies I and t (and therefore Q) were held constant. To obtain lower doses we use a technique of *ion dilution*: the electrolyte solution is intentionally diluted with quantities of a physiologically inactive cation. The quantity of active drug cations transported is then proportional to the mole fraction of active cations in the mixture.



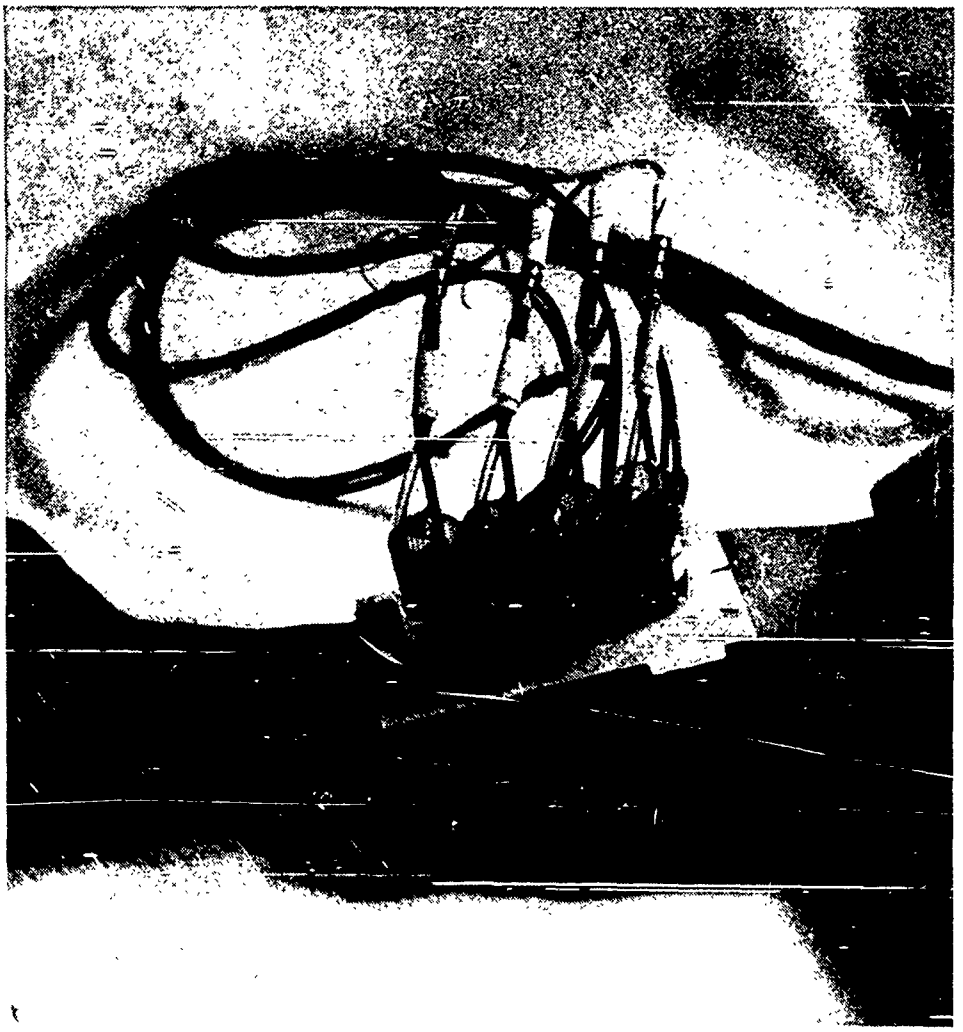
First, 20 mM stock solutions of active agents (acetyl-beta-methacholine, atropine sulfate, atropine methyl bromide, and scopolamine hydrobromide) and inactive agent (NaCl) were prepared. Then, measured amounts of active agent and NaCl stock were mixed in 20 ml vials to create the treatment solutions containing equal numbers of ions but differing in their ratio of active to inactive ions.



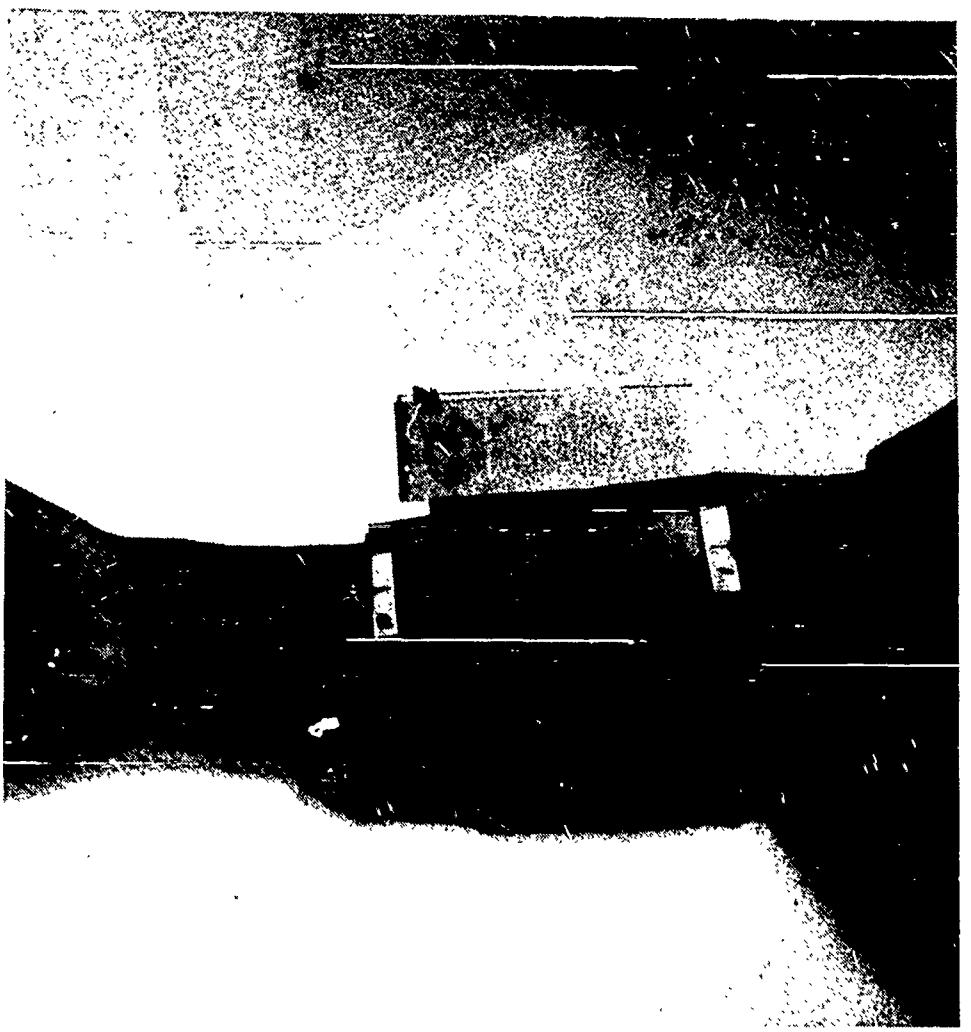
All apparatus was designed to study simultaneously 4 treatment areas on the mid volar surface of the forearm. Test sites were 2.1 cm D circles, 2.6 cm apart. A plastic guide with velcro fasteners (a) deliniated the treatment area and assured proper apparatus alignment. Iontophoresis apparatus consisted of 4 independent battery operated constant-current sources. The cathode assembly (b) consisted of 4 "Red Dot" disposable ECG electrodes. The anode assembly (c) consisted of 4 Ag-AgCl electrodes and filter pads that fit into machined wells 2.1 cm D and 0.1 cm deep. The filter pads were saturated with 0.2 ml of the appropriate pretreatment or treatment solution.



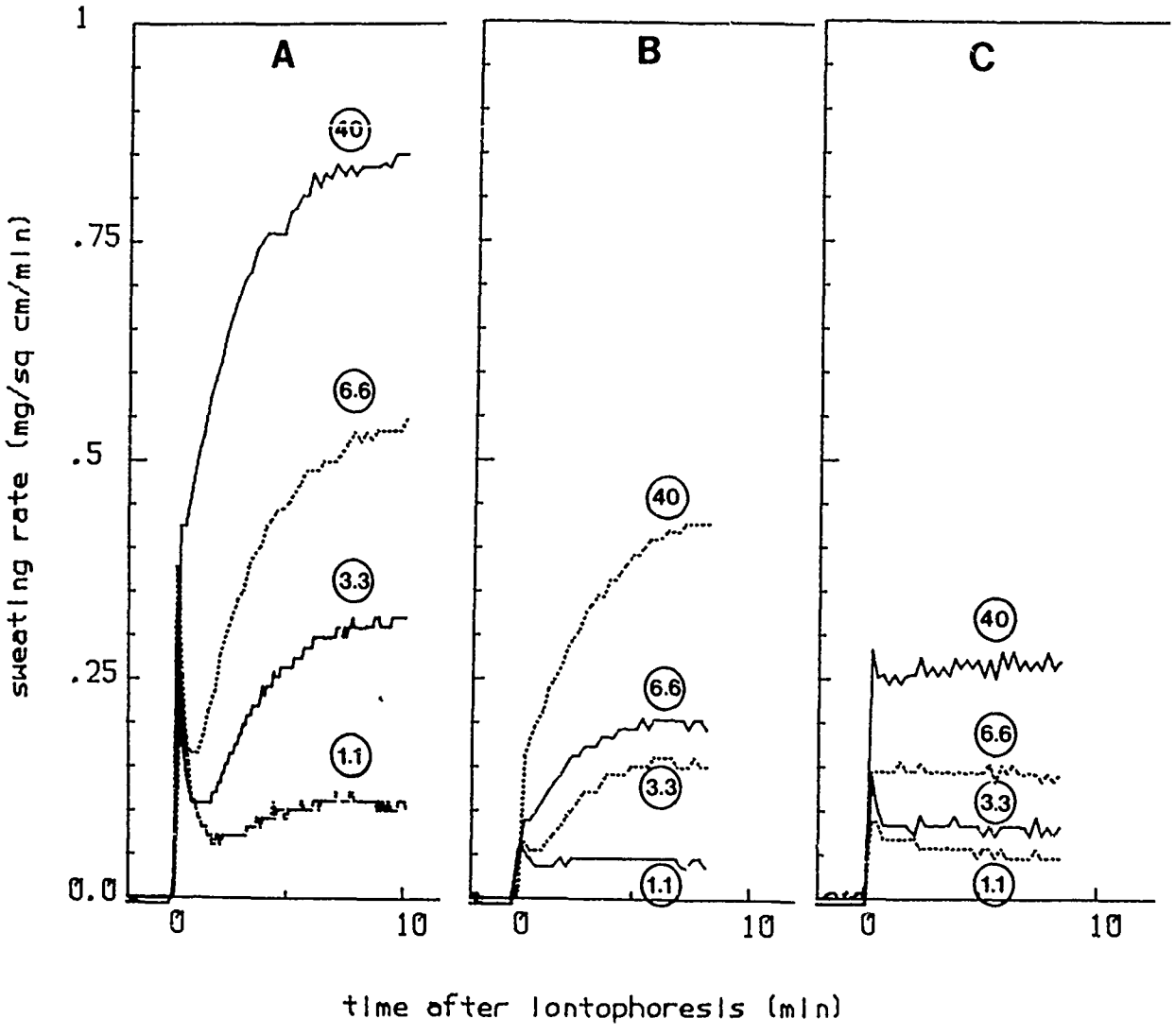
The cathode assembly was mounted on the forearm directly opposite the anode assembly. For pretreatment, the electrode assembly was placed into the template and the current sources were rapidly brought to the prescribed level and maintained for the prescribed time. Following pretreatment, skin and electrodes were wiped dry and the procedure repeated with treatment solutions.



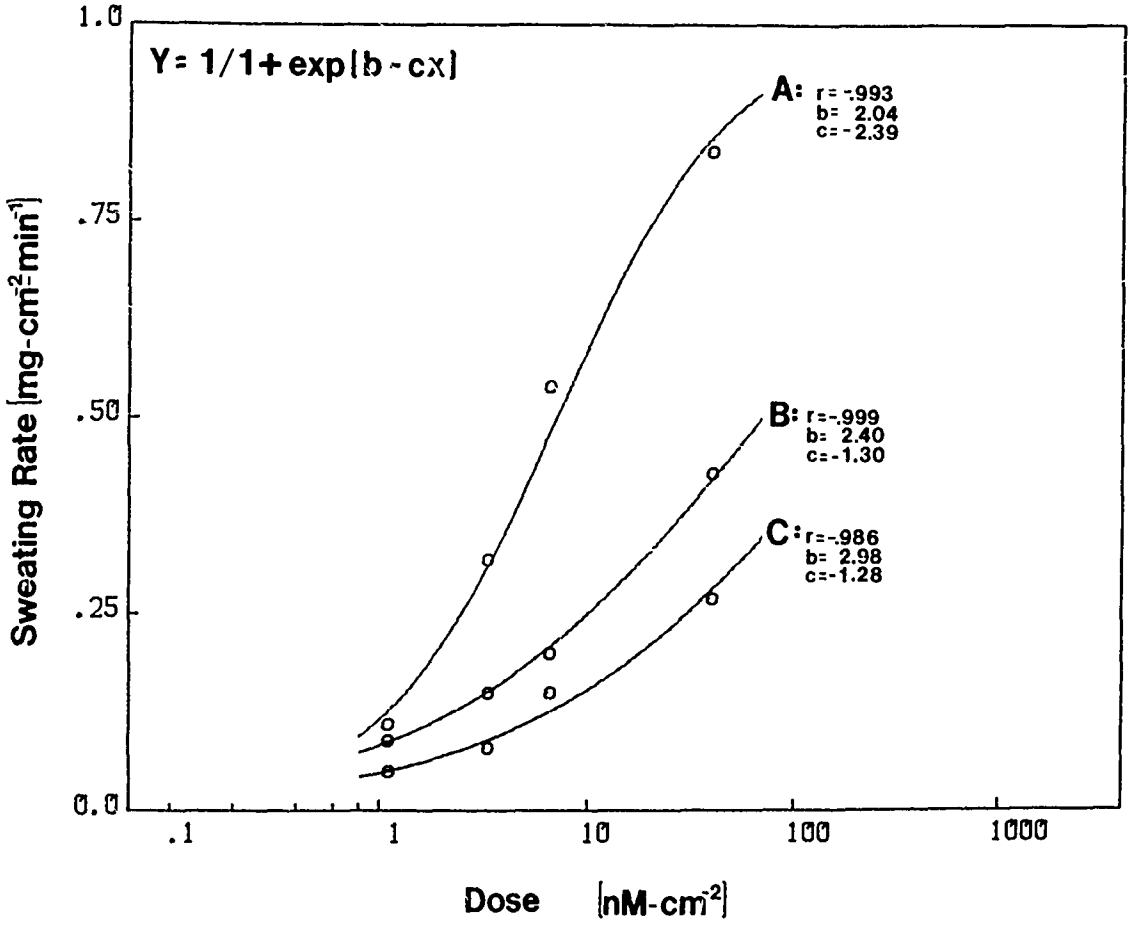
Immediately following treatment an assembly of 4 evaporative sweat capsules, whose centers aligned with the treatment centers, were positioned on the sites and secured by velcro fasteners on the template. Exposed capsule area was 18% smaller than the treatment area to avoid possible edge effects. Inside each capsule dry nitrogen gas was directed uniformly over the skin at 200 ml/min; this flow was sufficient to evaporate sweat as soon as it appears on the skin surface. Capsules had small fast-responding capacitance relative humidity sensors. Analog voltages were automatically digitized every 5 sec and stored on computer disk.



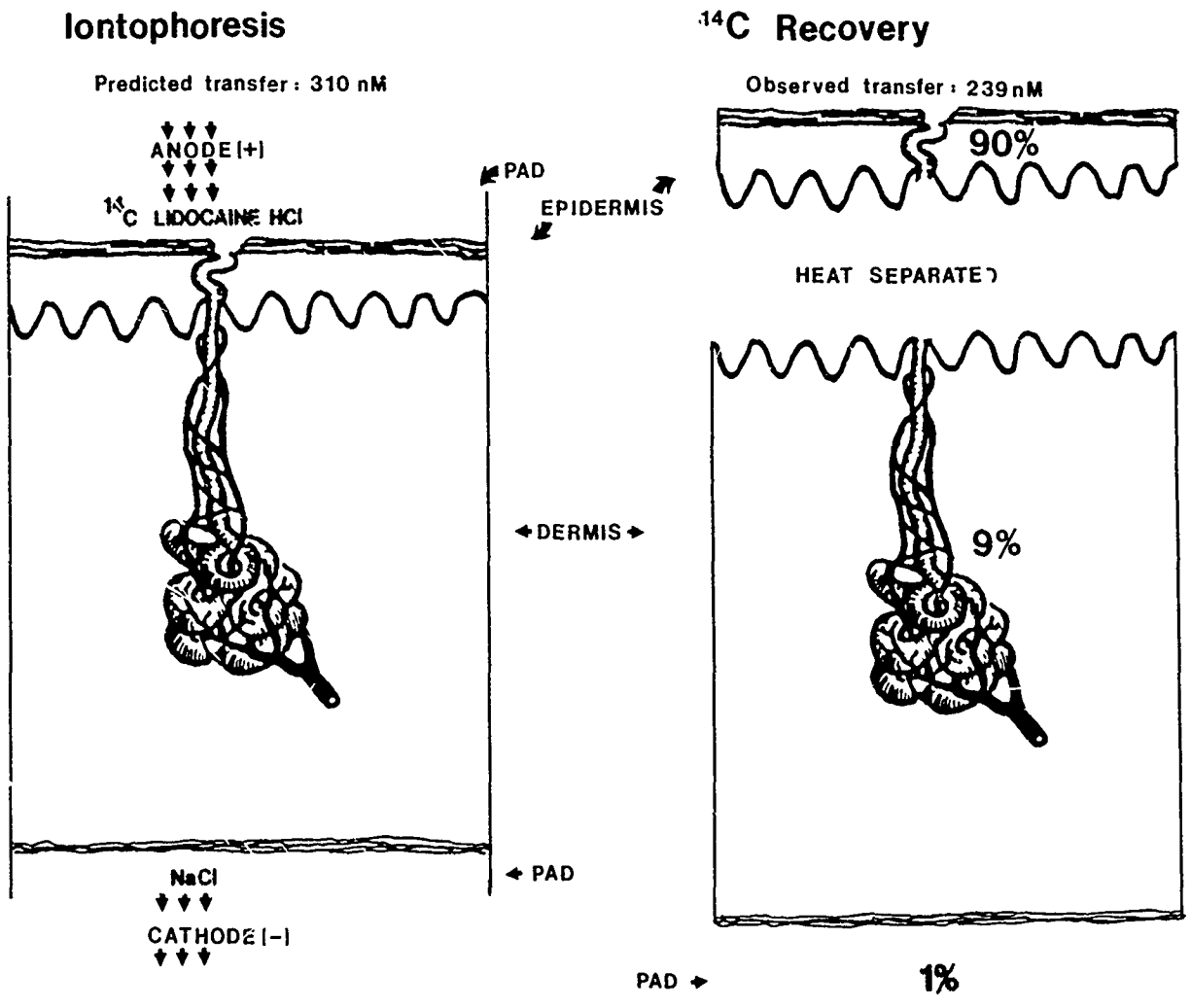
In many experiments the sweat capsules were removed as soon as peak heights had registered and impressions of active glands were made using iodinated paper. Counts of active glands under each capsule area were made with the aid of a counting grid and a low-power surface microscope.



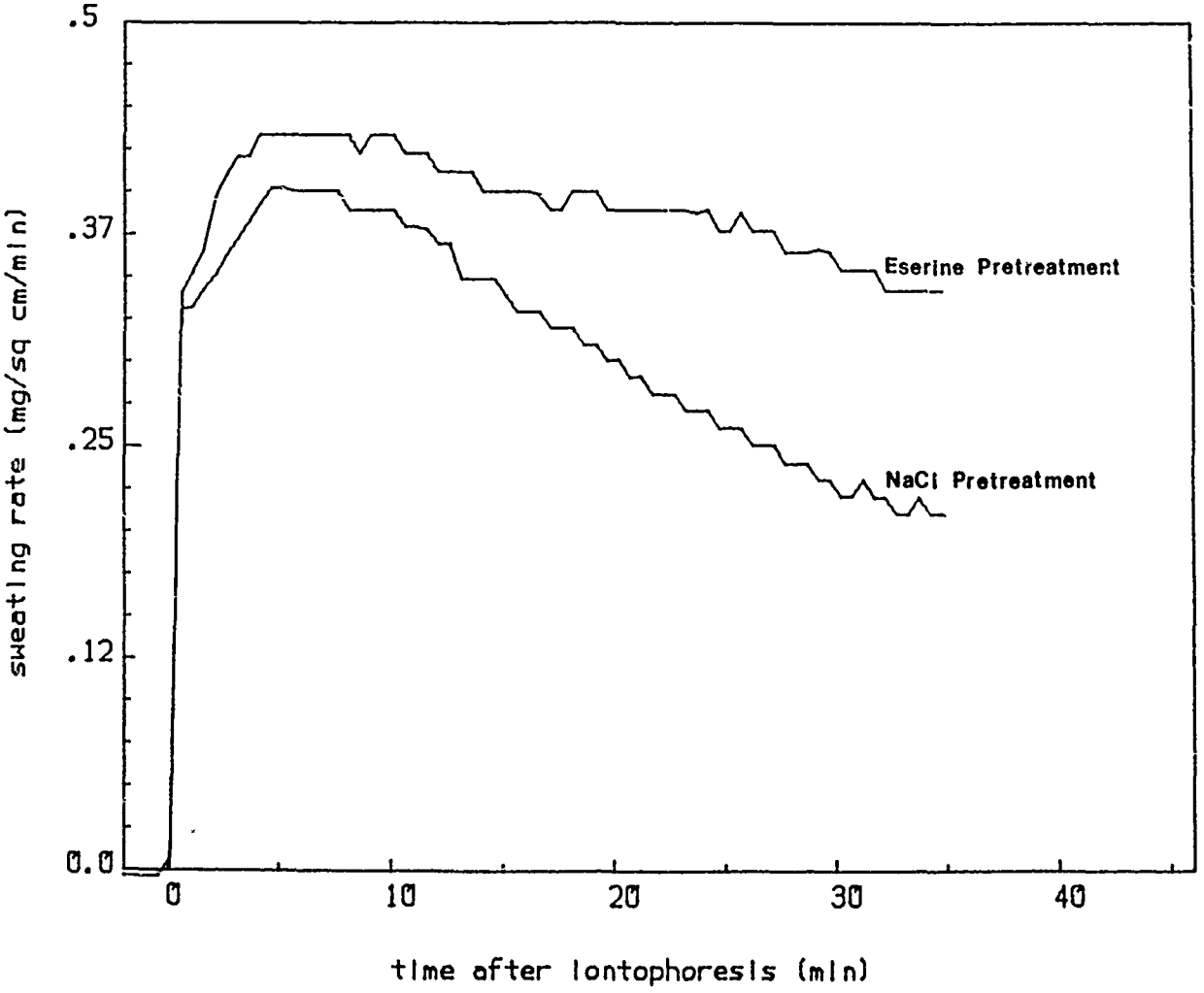
Data from methacholine dose-response tests on 3 subjects illustrate the variety of responses to the same doses (*circled numbers are calculated doses in nM/sq cm*). Typically, sweat rate climbed for 8-10 min and plateaued at a peak value that lasted for 2-3 min (*Panels A & B*), but in a few subjects the peak was achieved almost immediately and remained for as long as 7-8 min (*Panel C*). The peak height was defined as the tissue response. All subjects showed a slow exponential decline to baseline (not recorded). At high doses, measurable sweating was evident for up to an hour.



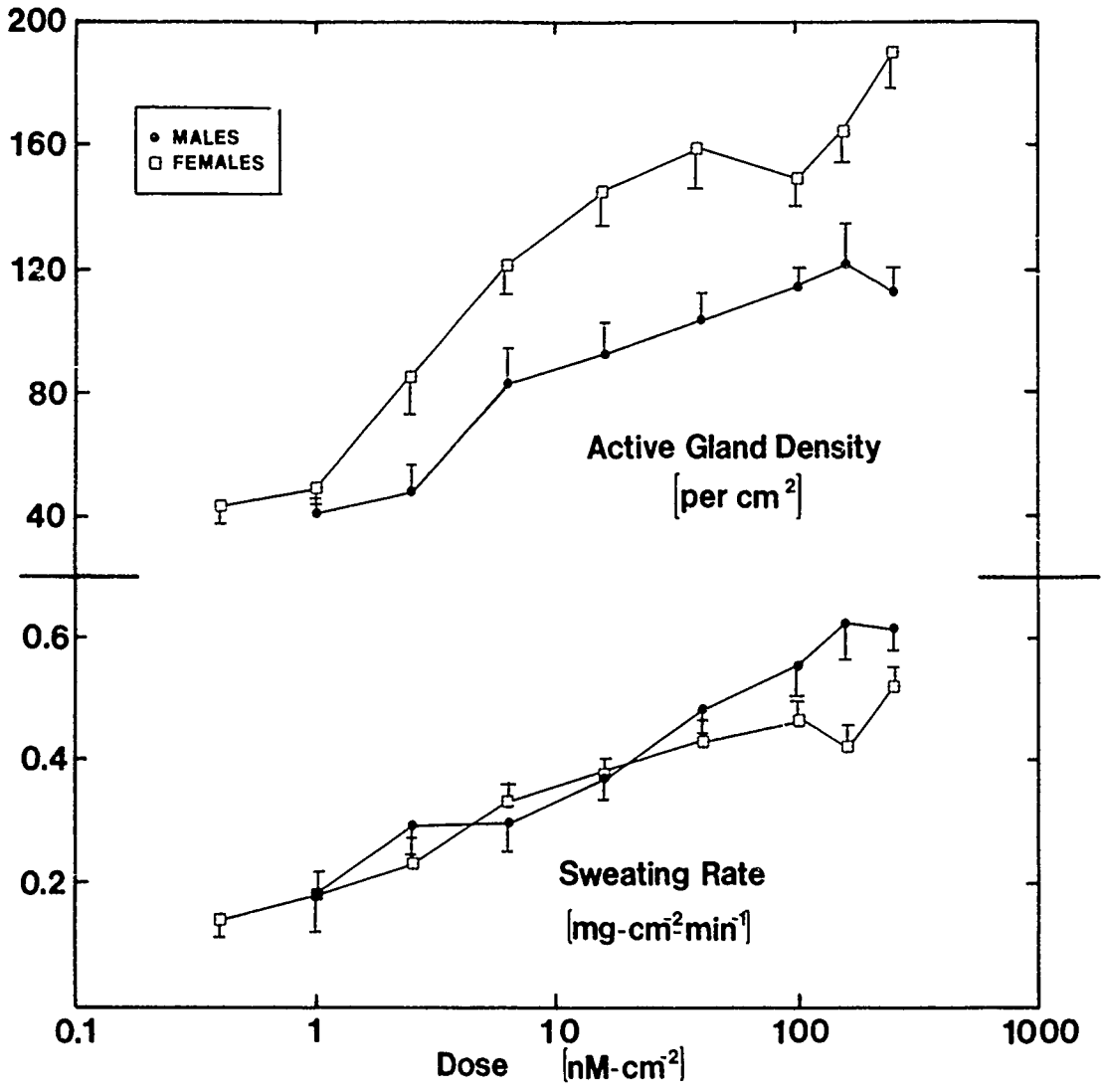
Individual peak sweating responses were hyperbolically related to the calculated methacholine dose. Here peak data from panels in the previous figure are plotted as a function of log dose. Best-fitting curve segments through these points were determined by a least squares method (Foster, K. G., J. Physiol. 213: 277-290, 1971). Subjects varied in both "shift" (*b*) and "shape" (*c*) parameters.



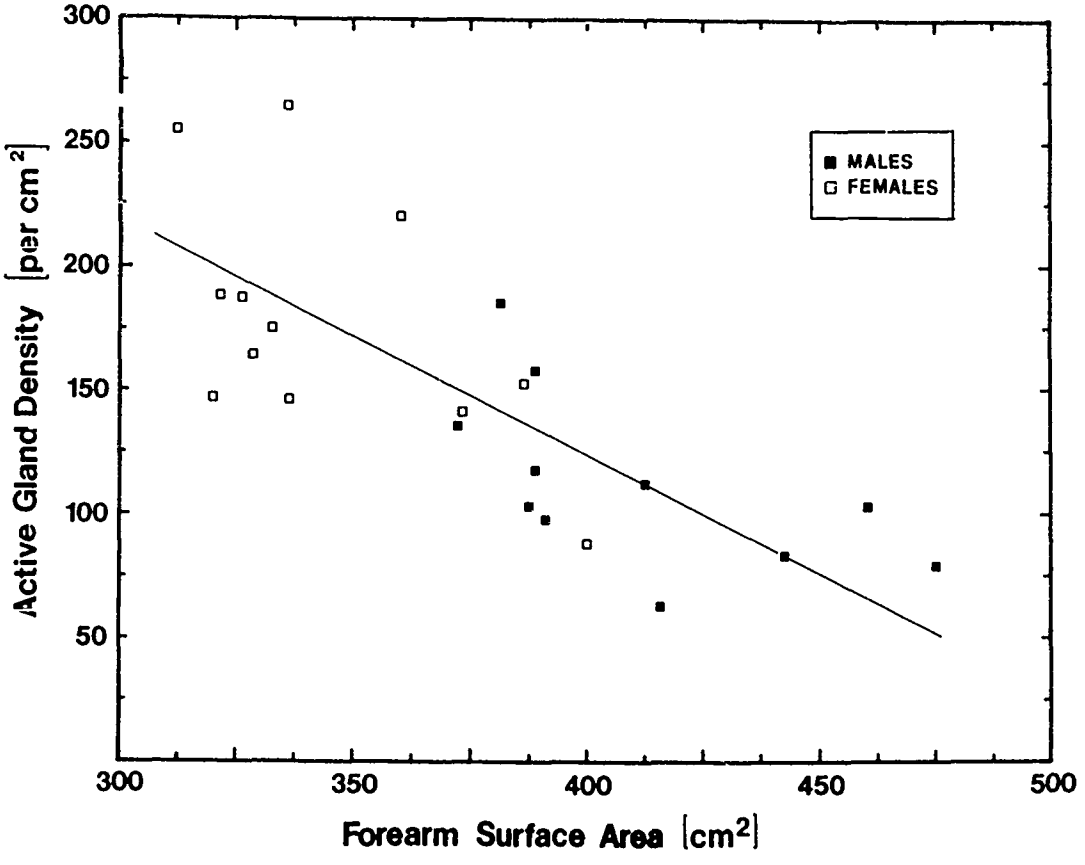
Drawing illustrating a pilot experiment on the iontophoretic transfer of a small labeled molecule (C-14 labeled lidocaine-HCl) through 5 samples of fresh autopsy skin. The molecular weight of lidocaine is similar to mecholyl, and both should have roughly the same electrophoretic mobility. Most of the label remained in the epidermis; only 9% of labeled cations actually reached the dermis and only 1% passed through the dermis. More work of this type is needed so that periglandular concentration can be estimated from delivered dose.



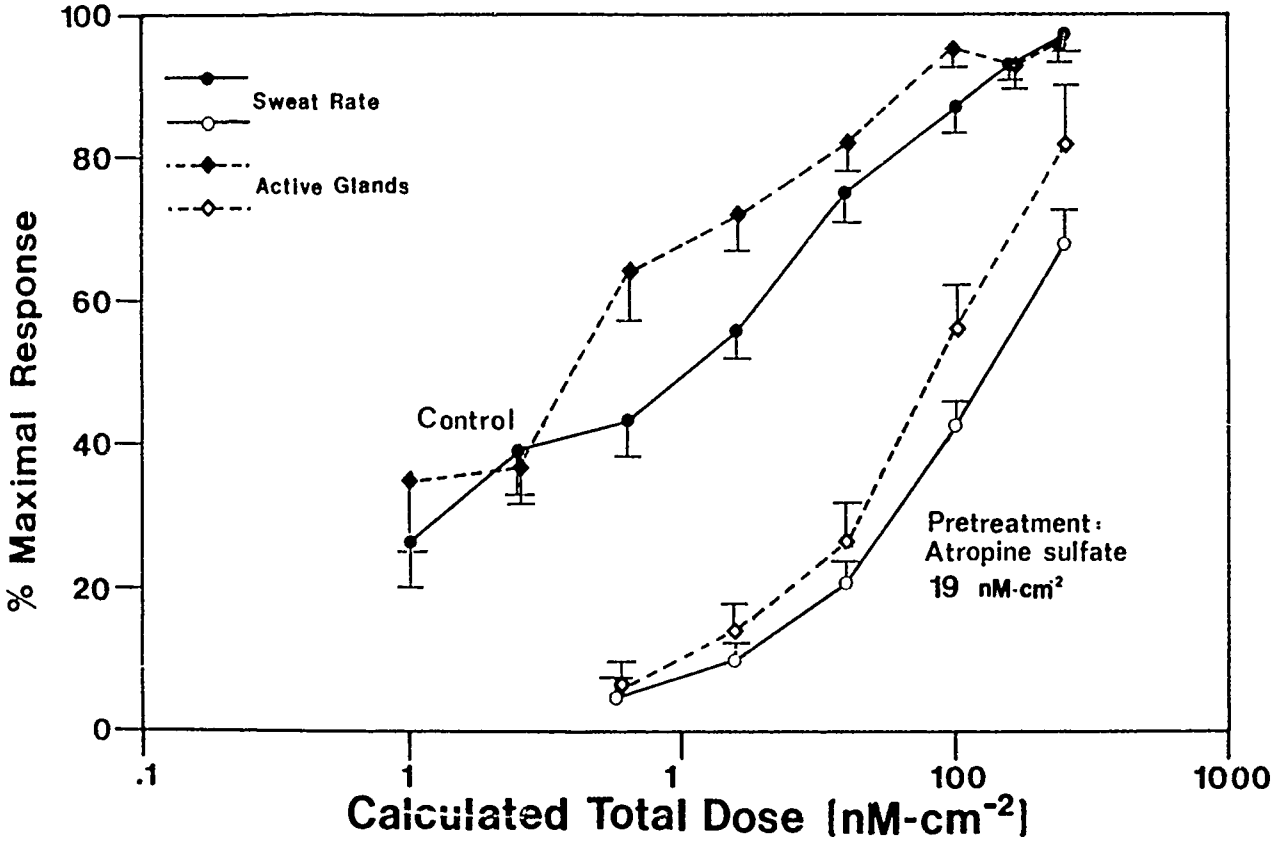
This figure shows the effect of pretreatment with a cholinesterase inhibitor on the sweating rate response to moderate stimulation with methacholine. Peak time is essentially the same for both control and treated sites, but on the treated site the rate of decay from peak is much slower. This indicates that enzymatic degradation is a major factor contributing to the decline from peak sweating rate. Further studies will be needed to determine whether this non-invasive test can reflect plasma cholinesterase levels.



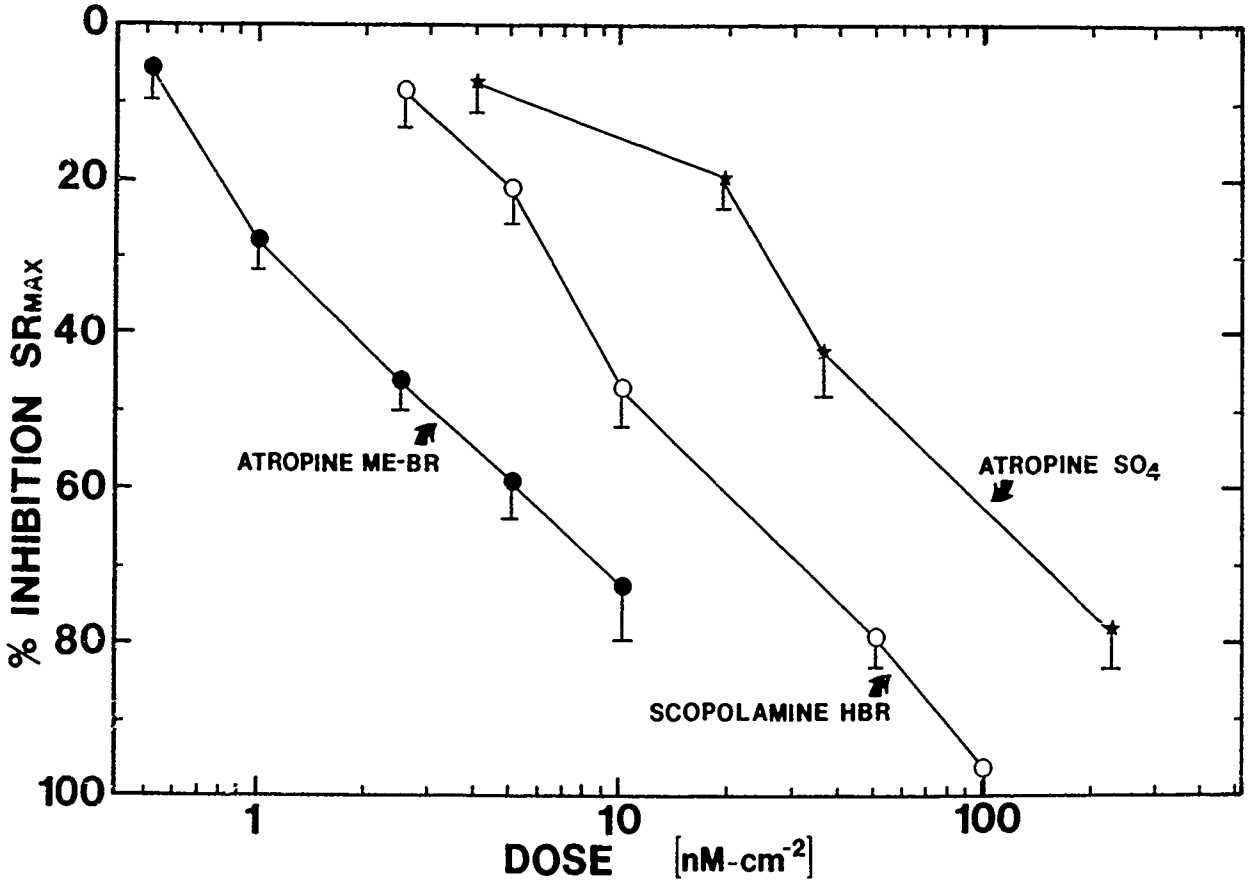
This figure shows sweating rate and active gland counts averaged from data on 12 male and 12 female subjects using methacholine doses of 0.6 to 250 nM/sq cm. In the control curves (NaCl pretreatment) for both males and females, increments in sweating rate were accompanied by an increase in the number of active glands. However, at the same dose of agonist, average active gland density was higher in female subjects but average peak sweating rate was nearly the same in both males and females.



Maximal active gland density was correlated to computed forearm surface area. Males tended to have both a greater forearm surface area and a lower maximal gland density than females. Since the sweating rates were similar, males must have a higher maximal output per gland than females.

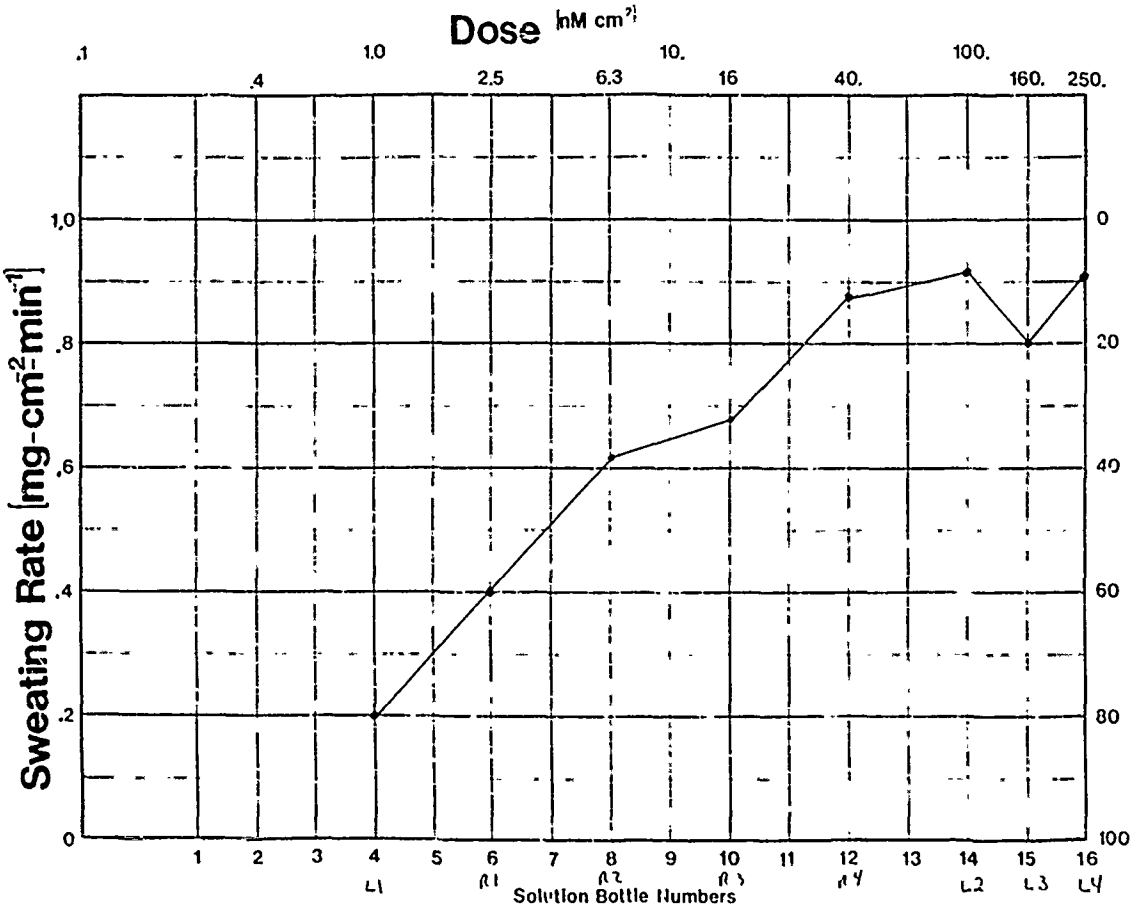


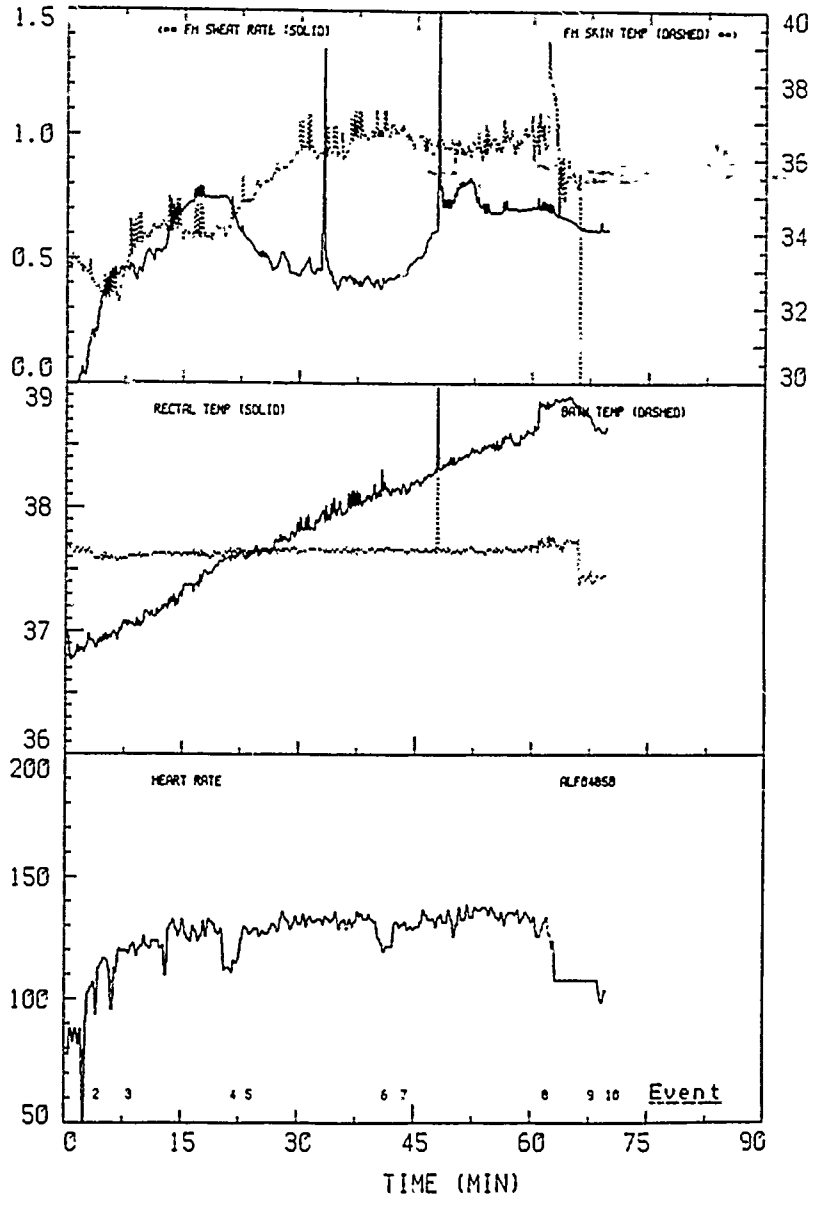
Average results of atropine sulfate assay on 12 male subjects expressed as percent maximal response. Antagonist in this concentration (19 nM per sq cm) shifted the response curve by more than 1 log unit to the right. Note correspondence between peak sweat rate and active gland density.



Percent inhibition of maximal sweating rate as a function of antagonist dose in 12 male subjects. All antagonists administered by pretreatment. At 50% inhibition the ED-50's for atropine methyl bromide, scopolamine hydrobromide, and atropine sulfate were 3, 12, and 54 nM per sq cm, respectively. The potency ratios for scopolamine hydrobromide and atropine methyl bromide (compared to atropine sulfate) were 4.5 and 18, respectively.

We have recently started a study to determine if a person's heat tolerance can be predicted from a room temperature test of the cholinergic responsiveness of their sweat glands; example data are shown below. First, a standard forearm cholinergic assay is done at room temperature (left). This result will be compared with outcomes of 2 other independent heat tolerance tests: a controlled hyperthermia immersion test (right) and a heat tolerance step test (result not shown).





Time	Event	Description
0.0	1	Start immersion
3.0	2	Start Pedalling
7.1	3	Capsule heater ON
20.6	4	Rest Stop
22.6	5	Start pedalling Oxygen Consumption
40.2	6	Rest Stop Capsule strap loosened
42.7	7	Start Pedalling
60.9	8	End immersion Capsule heater off HR monitor off
66.6	9	Skin Temp off
69.0	10	HR monitor back on
71.0	--	Expt ended

Total Fluid Loss: 1180 g. Ave sweat rate: 998 g/hr.

EFFECTS OF ORGANOPHOSPHATE CHOLINESTERASE INHIBITORS ON HYPOTHALAMO-PITUITARY-ADRENAL (HPA) ACTIVITY AND CORE TEMPERATURE

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Department of Pharmacology, UCLA

INTRODUCTION

The specific objectives of this research proposal are to determine the effects of two irreversible organophosphate (OP) cholinesterase (ChE) inhibitors on neuroendocrine function. The specific compounds to be examined are the extensively studied OP, diisopropyl-fluorophosphate (DFP), and the extremely potent OP, soman. A survey of the literature has shown that little is known about the effects of OP ChE inhibitors on neuroendocrine function. However, the available evidence showing cholinergic effects on hormone release from the anterior pituitary gland indicates that irreversible OP inhibitors likewise would alter the normal patterns of hormone secretion. It is of importance to determine the neuroendocrine effects of OP agents because hormones, in particular the hypothalamus-pituitary-adrenal (HPA) axis, play a primary role in the regulation of homeostasis . Since many of the adaptive responses of the organism to changes in its external and/or internal environment are mediated by hormones and coordinated by the central nervous system (CNS), it is also possible that OP induced perturbations of hormone secretion may have biological effects that would not be predicted from the inhibition of ChE alone.

Although there is abundant evidence that acute or chronic administration of OP cholinesterase inhibitors interfere with cholinergic function, little is known about the extent to which neuroendocrine function is disrupted as a result of this interference. We propose to investigate the effects of OP agents on neuroendocrine function and also to study the effects on body temperature because the centers for thermoregulation are also in the hypothalamus, and changes of core temperature are readily measured by noninvasive procedures.

METHODS

All the experiments were done on rats which is likely the animal most suitable for elucidating the mechanisms underlying the effects of acute and chronic OP exposure on neuroendocrine function. In the initial studies rats were injected with DFP, 0.75 mg/kg to 2.0 mg/kg, and were decapitated at varying intervals after injection, trunk blood was collected for hormone assay and brains removed for cholinesterase assay. The effect of OP on HPA activity was determined by measuring the changes of plasma corticosterone concentration following injection. Plasma corticosterone was measured by a fluorometric method (J. Lab. Clin. Med. 53:830, 1959) and AChE activity by a colorimetric method (Biochem. Pharmacol. 7:88,1961). The remaining plasma was stored frozen for radioimmunoassay of prolactin, TSH and growth hormone. The effect of OP inhibitors on body temperature was determined by measuring core temperature at 30, 60, 90, 120, 150 and 180 min after injection. The hypothermic index, the area under the temperature response curve, was used to assess the effects of soman over the 180 min period. The statistical significance of differences between group means was determined by the Student t test.

Fig. 1. Effects of DFP on brain AChE activity and plasma corticosterone levels.

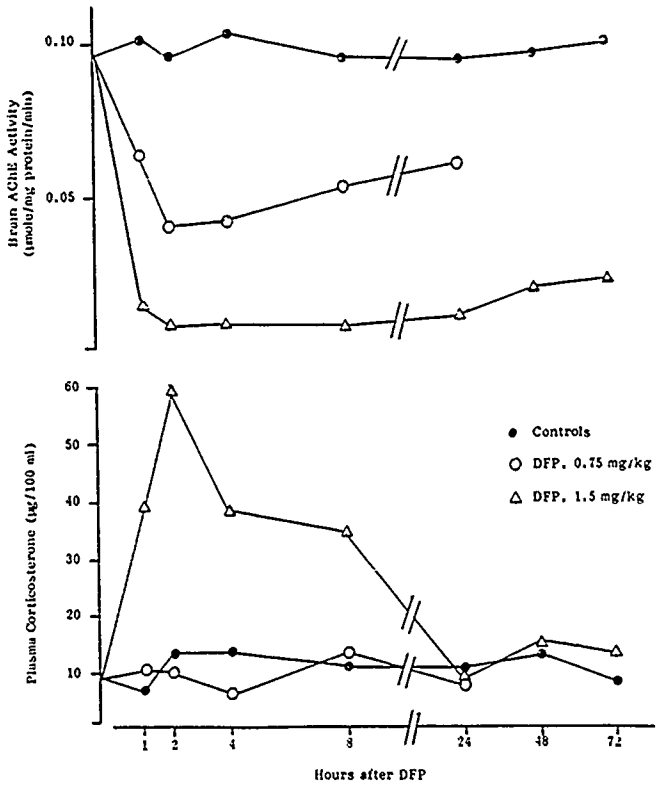

Fig. 1. The acute effects of DFP, 0.75 and 1.5 mg/kg s.c., on brain cholinesterase activity and plasma corticosterone concentrations were compared with those observed following injection of the diluent, peanut oil. With DFP, 0.75 mg/kg, whole brain cholinesterase activity was reduced below 50% of control but plasma corticosterone concentration was not changed. With 1.5 mg/kg brain cholinesterase activity was reduced below 10% and plasma corticosterone concentration was increased 6 fold at 2 h. Plasma corticosterone levels then declined to control values at 24 h while brain cholinesterase activity remained suppressed, indicating adaptation of the HPA axis to the low level of enzyme activity. Postsynaptic muscarinic receptor subsensitivity was not the basis of adaptation because oxotremorine, 0.125 mg/kg i.p., produced the same increases of plasma corticosterone concentration in DFP rats at 24 h as in the diluent treated controls.

Fig. 2. Relationship between inhibition of hypothalamic AChE and activation of HPA axis following DFP.

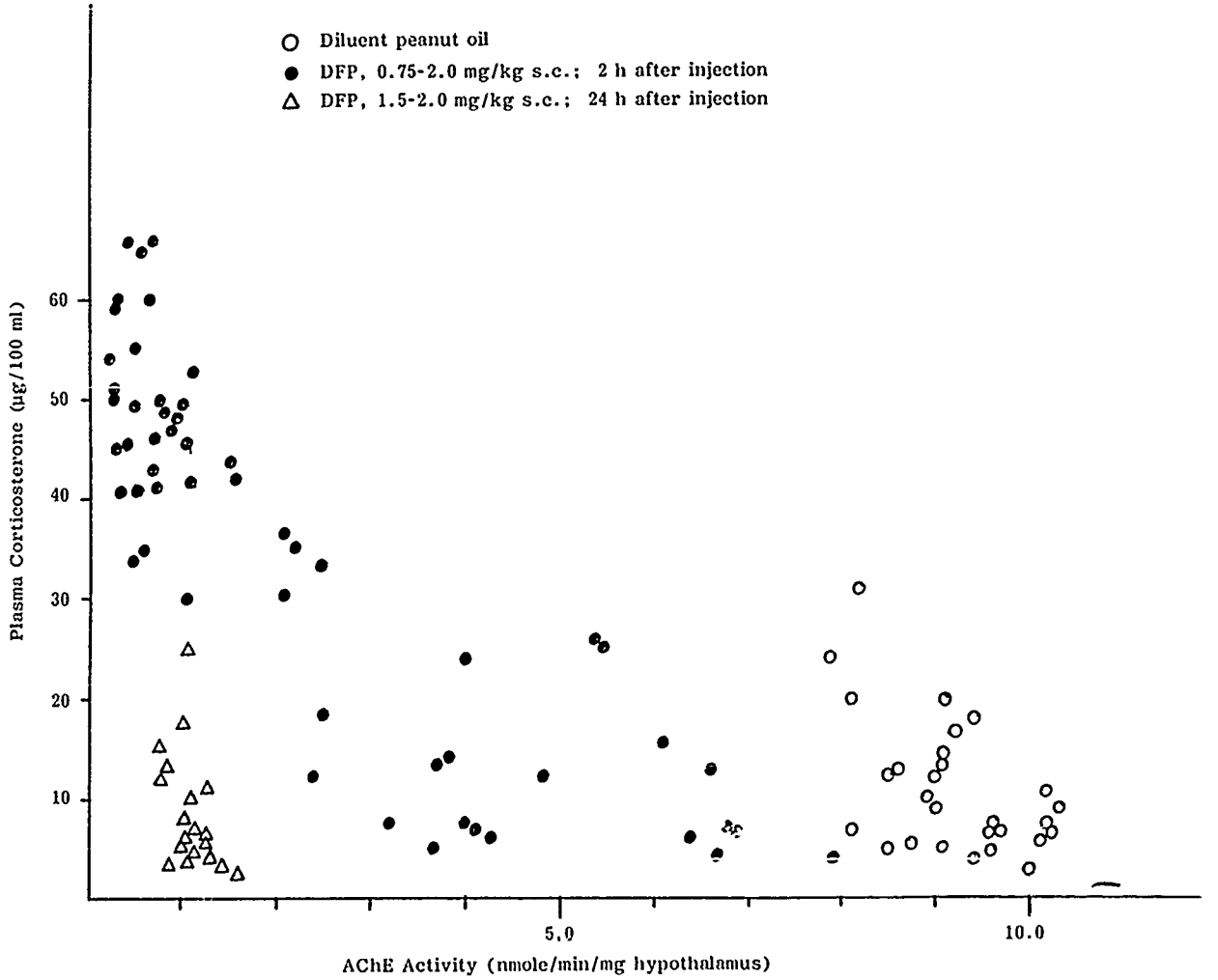

Fig. 2. Rats were injected with DFP, 0.75, 1.25, 1.5 and 2.0 mg/kg, and hypothalamic AChE activity and plasma corticosterone concentration assayed at 2 h. The results show that the acute DFP induced increases of plasma corticosterone concentration, indicating stimulation of HPA activity, was related to the degree to which hypothalamic AChE was inhibited. DFP doses producing less than 75% inhibition had no significant effect on plasma corticosterone. Diluent treated normal rats, in contrast, had low plasma corticosterone levels and high AChE activity while DFP adapted rats at 24 h had low levels of both plasma corticosterone and hypothalamic AChE.

Fig. 3. DFP tolerance : Effects of chronic administration of DFP on hypothalamic AChE and HPA activity.

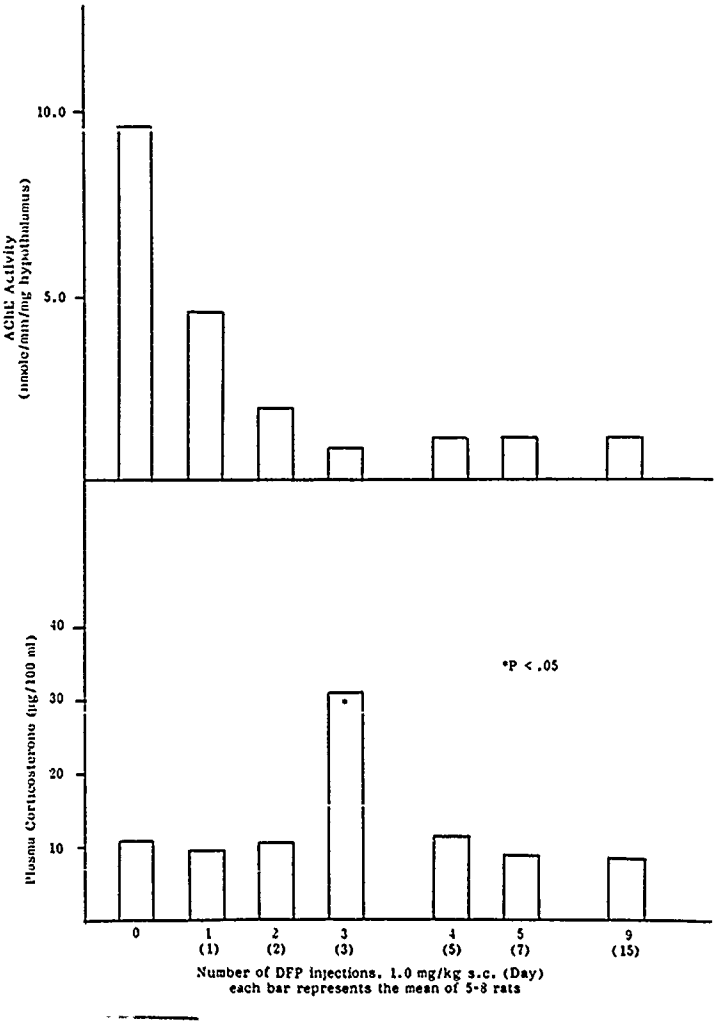

Fig. 3. The effect of chronic administration of DFP on HPA activity was assessed to determine the rate and magnitude of the development of tolerance. The low dose of DFP, 0.75 mg/kg, which has no effect on plasma corticosterone concentration was administered daily to determine the cumulative effects of subacute, chronic treatment. Fig. 3 shows that following the third injection of DFP plasma corticosterone was significantly elevated and hypothalamic AChE activity was reduced to 15% of normal, suggesting again that a critical level of inhibition was required for DFP activation of the HPA axis. After the third day DFP was injected on alternate days. As the injections were continued DFP tolerance developed rapidly and persisted throughout the period of treatment.

Table 1

Effect of chronic DFP treatment on HPA response to cholinergic agonists

	DFP injection, day		
	0	8	14
	Plasma corticosterone (μg/100 ml)		
saline	15.4 ± 3.0 (8)	17.6 ± 3.0 (7)	13.8 ± 3.2 (5)
oxotremorine (0.125 mg/kg)	50.0 ± 2.0 (7)	43.7 ± 7.3 (6)	34.3 ± 2.9 (7)
physostigmine (0.125 mg/kg)	38 ± 4 (5)	42.4 ± 3.9 (5)	31.4 ± 4.2 (5)
nicotine (0.5 mg/kg)	35 ± 1.0 (5)	*8.1 ± 2.0 (7)	

( ) number of animals

*P < .05 vs Day 0

Table 1. The objective of these experiments was to assess the involvement of muscarinic and nicotinic receptors in the development of tolerance to the effects of OP on HPA activity. Rats were injected chronically with DFP as shown in Fig. 3 then tested with oxotremorine, physostigmine or nicotine at 24 h after the last injection of DFP. The results show that sensitivity to oxotremorine was not altered at Day 8 but was significantly reduced by Day 14, showing that muscarinic receptor subsensitivity was present following the longer course of DFP treatment. In contrast subsensitivity to physostigmine was not observed at Day 8 or 14 of DFP treatment. Complete tolerance to nicotine was present at Day 8 as shown by its failure to raise plasma corticosterone levels of DFP treated rats.

Fig. 4. Development of tolerance to DFP induced hypothermia.

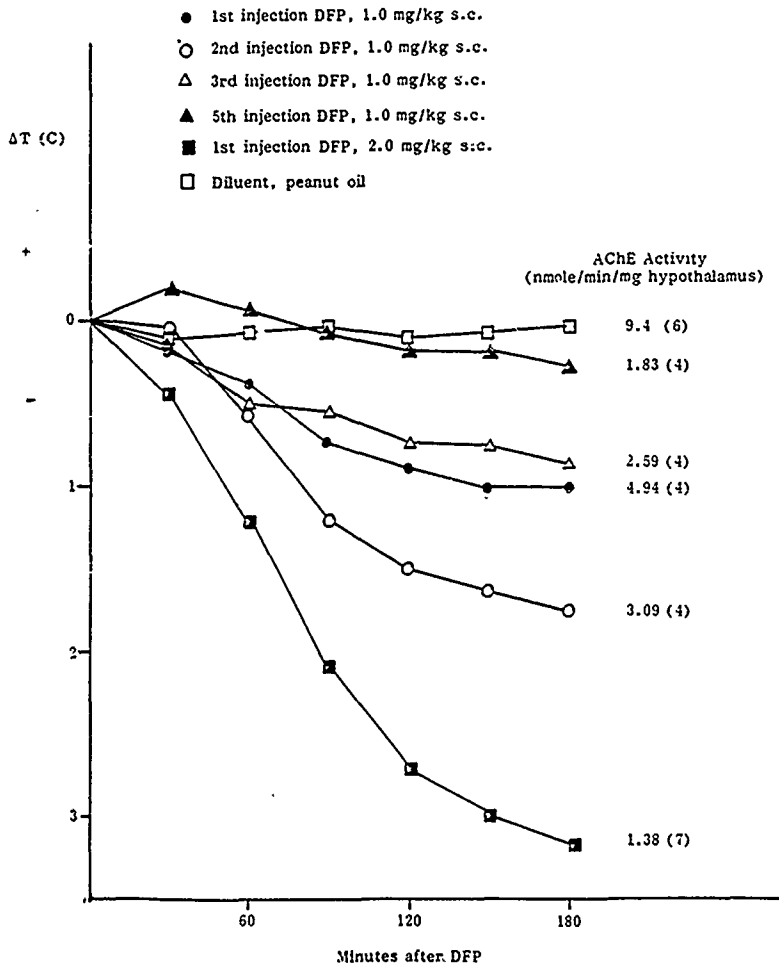

Fig. 4. In the experiments to determine the effects of chronic DFP treatment on body temperature, rats were injected with DFP, 1.0 mg/kg, every 48 h. Core temperature was recorded for 3 h and 4 rats were sacrificed for AChE assay after each injection of DFP. Following the 1st dose of DFP core temperature declined over 3 h and hypothalamic AChE activity was reduced approximately 50%. The hypothermic response was greater after the 2nd dose as was the inhibition of AChE activity. By the 3rd injection there was evidence of tolerance, and by the 5th dose no fall in body temperature was seen, at the time hypothalamic AChE activity was lowest. The greatest hypothermia was induced acutely by DFP, 2.0 mg/kg, which produced inhibition of hypothalamic AChE activity comparable to that observed following 5 injections of DFP, 1.0 mg/kg.

Fig. 5. Effects of oxotremorine on body temperature of DFP tolerant rats.

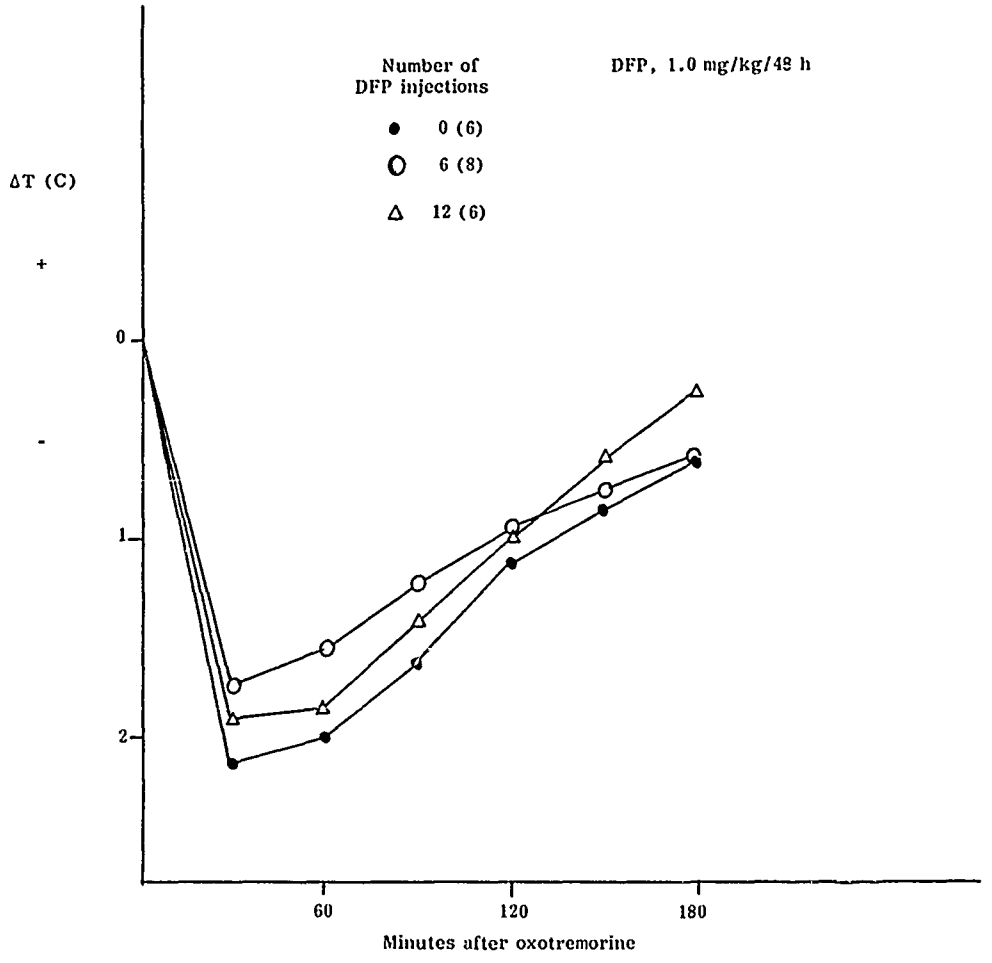

Fig. 5. The goal of this experiment was to determine if the development of tolerance to the hypothermic effect of DFP was a consequence of diminished sensitivity of the cholinergic receptor following chronic administration of DFP. The cholinergic agonist oxotremorine, 0.40 mg/kg s.c., was used to induce a hypothermic response. In the control DFP naive rats oxotremorine caused a fall in core temperature of 2.3±0.3°C at 30 min. DFP treated rats tested after the 6th and 12th injection had corresponding falls in temperature of 1.75±0.2 and 1.92±0.3°C, of which neither was significantly different from the untreated controls. These findings suggest that the development of tolerance to the hypothermic action of DFP was not related to decreased sensitivity of the postsynaptic cholinergic receptor.

Fig. 6. Dose response effects of soman on body temperature.

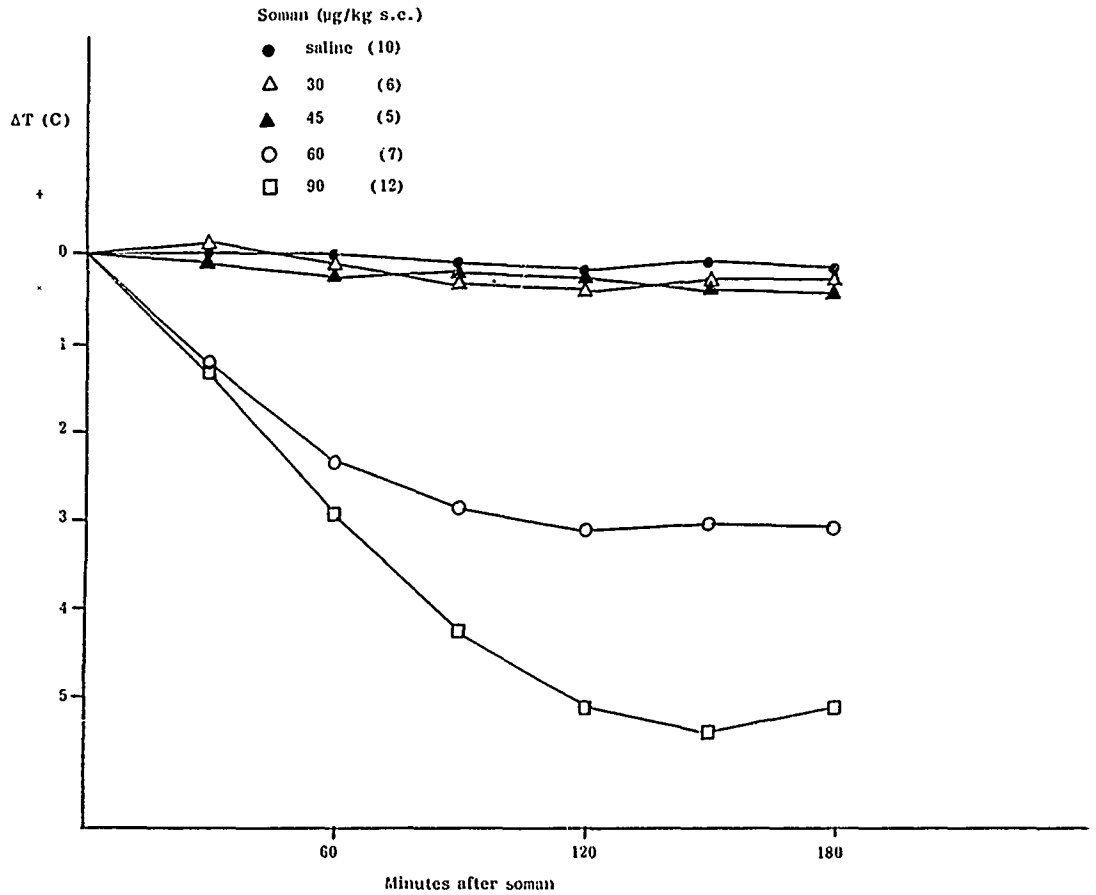

Fig. 6. A dose response study was done with soman, 30 to 90 µg/kg s.c., to determine its effect on body temperature and hypothalamic AChE activity under our laboratory conditions. Core temperature was measured for 3 h and the rats were observed continuously during this time for changes of behavior. At 4 h the rats were sacrificed and the brains removed for assay of AChE activity. The time course of the temperature changes is shown in Fig. 6. Soman, 30 and 45 µg/kg, had no significant effects on body temperature. Soman, 60 µg/kg, had variable effects on temperature in which 2 rats showed little decrease (less than 0.7°C), 2 rats a moderate decrease (less than 2.8°C) and 3 rats a severe hypothermia (greater than 5.0°C). Soman, 90 µg/kg, induced the greatest fall of core temperature as well as effects on behavior characterized by ataxia, tremor, spasms and convulsions. These abnormal behaviors, although less intense, were also manifest in the 3 rats exhibiting severe hypothermia following the 60 µg/kg dose.

Fig. 7. Relationship between inhibition of hypothalamic AChE activity and decrease of core temperature following soman.

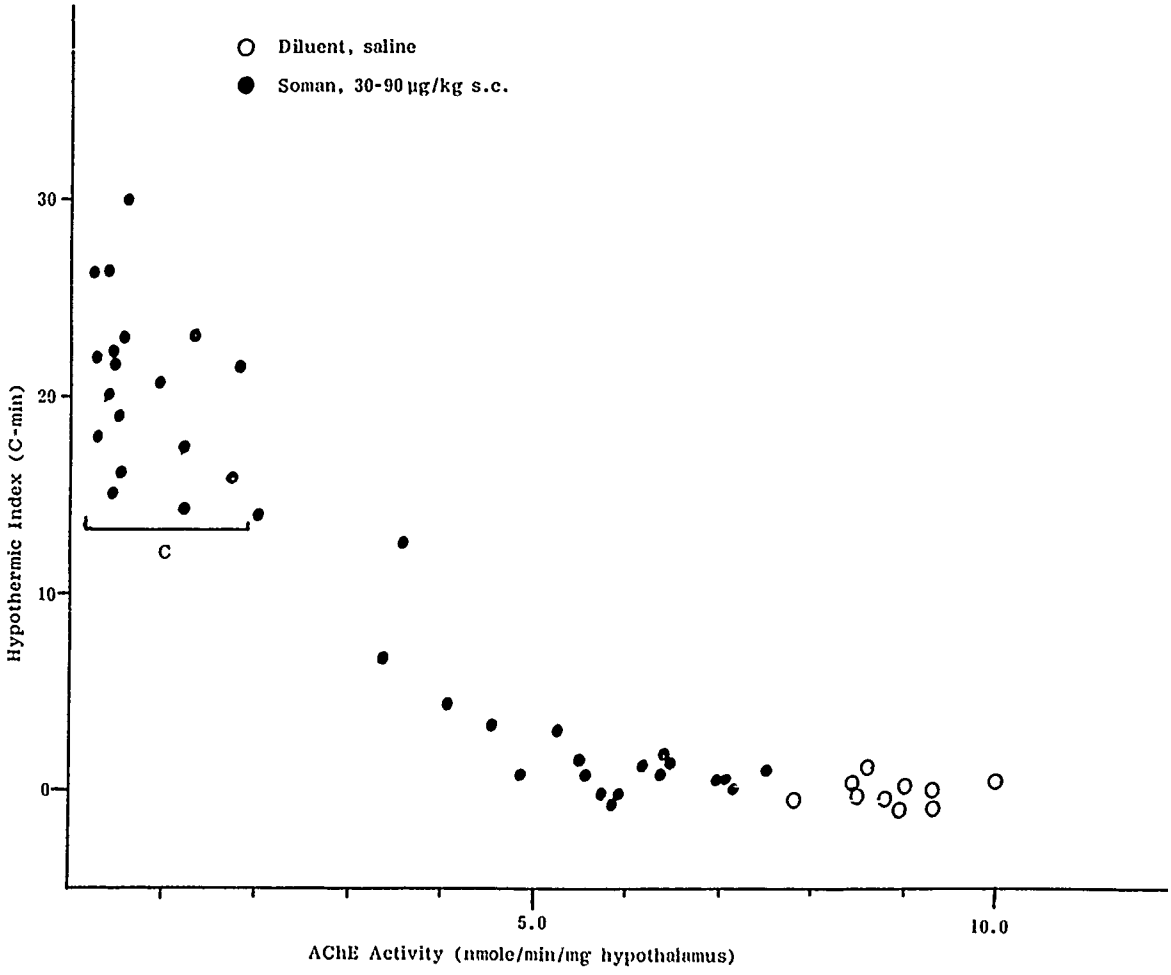

Fig. 7. The relationship of the fall in body temperature, expressed as the hypothermic index (the area under the temperature curve), to the inhibition of hypothalamic AChE activity and to the occurrence of seizures following exposure to soman are shown in Fig. 7. Marked hypothermia, indicated by a high hypothermic index, was observed following soman inhibition of AChE activity to 20% of normal levels. Convulsions, indicated by C, almost invariably developed in rats with a high hypothermic index. Inhibition of hypothalamic AChE to the same extent by DFP was not correlated with convulsions or profound hypothermia, suggesting that the effects of soman in part may be mediated by actions other than the inhibition of cholinesterase.

CONCLUSIONS

1. A relationship between DFP inhibition of hypothalamic AChE activity and stimulation of HPA activity has been shown.

2. Tolerance develops to DFP activation of the HPA axis and appears to involve subsensitivity to both muscarinic and nicotinic stimulation.

3. Tolerance develops to DFP induced hypothermia but muscarinic subsensitivity to the temperature response could not be shown.

4. Dose- response studies of the effects of soman on body temperature showed that the greatest hypothermic responses were associated with the presence of seizure activity.

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5. Enzymes for Protection and Decontamination

SELECTIVITY OF ORGANOPHOSPHINATE PRETREATMENT AGENTS WITH REGARD TO XENOBIOTIC-DEGRADING HYDROLASES

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Clemson University

ORGANOPHOSPHINATE PRETREATMENT IS INTENDED TO CAUSE TRANSIENT INHIBITION OF ACETYLCHOLINESTERASE FOR THE PURPOSE OF PREVENTING IRREVERSIBLE INHIBITION BY CHEMICAL WARFARE AGENTS. PHOSPHINYLATION OF ACETYLCHOLINESTERASE WILL BE ACCOMPANIED BY PHOSPHINYLATION OF OTHER SERINE HYDROLASES (B-ESTERASES) SUCH AS CARBOXYLESTER HYDROLASE. IN ADDITION, RELATED HYDROLASES SUCH AS ARYLESTER HYDROLASE WILL CATALYZE HYDROLYSIS OF CERTAIN ORGANOPHOSPHINATES.

WE ARE STUDYING TWO NON-TARGET ESTERASES, CARBOXYLESTERASE (A B-ESTERASE) AND ARYLESTER HYDROLASE (AN A-ESTERASE) FOR THEIR INTERACTION WITH ORGANOPHOSPHINATES. REACTIVATION OF CARBOXYLESTERASE INHIBITED WITH 13 DIFFERENT PHOSPHINATES WAS DETERMINED AND RANGED FROM 1.5% REACTIVATION IN 24 HOURS FOR 4-NITROPHENYL ISOPROPYL(PHENYL)PHOSPHINATE TO 65% REACTIVATION IN 24 HOURS FOR ENZYME INHIBITED WITH 4-NITROPHENYL METHYL(PHENYL)PHOSPHINATE. THE SAME 13 PHOSPHINATES WERE STUDIED WITH ARYLESTER HYDROLASE FROM RABBIT SERUM AND IT WAS FOUND THAT 10 OF THESE WERE GOOD SUBSTRATES FOR THE ENZYME. THIS WAS INDICATED BY THE FACT THAT MOST OF THESE COMPOUNDS HAD MEASURED Km VALUES LOWER THAN ETHYL PARAOXON AND MEASURED Vmax VALUES HIGHER THAN PARAOXON. THE Km VALUES RANGED FROM A HIGH OF 0.49 mM FOR 4-NITROPHENYL METHYL (TRIFLUOROMETHYLPHENYL)PHOSPHINATE TO A LOW OF 0.02 mM FOR 4-NITROPHENYL METHYL(2-FURYL)PHOSPHINATE.

WE HAVE EXPANDED THE STUDY OF SELECTIVITY TO INCLUDE THE STEREOSELECTIVITY OF HYDROLYSIS OF SOME OF THE CHIRAL PHOSPHINATES BY VARIOUS ENZYMES. WE HAVE EVIDENCE FOR STEREOSELECTIVE HYDROLYSIS OF 4-NITROPHENYL ETHYL(PHENYL)PHOSPHINATE BY RABBIT SERUM ARYLESTER HYDROLASE AND OF A STEREOSELECTIVE INTERACTION OF 4-NITROPHENYL ETHYL(PHENYL)- AND 4-NITROPHENYL ISOPROPYL(PHENYL)PHOSPHINATE WITH THE SERINE PROTEASES TRYPSIN AND CHYMOTRYPSIN.

STRUCTURE-ACTIVITY RELATIONSHIPS WILL BE DEVELOPED FOR THESE REACTIONS AND COMPARED WITH THOSE DEVELOPED BY OTHERS FOR INHIBITION OF ACETYLCHOLINESTERASE BY ORGANOPHOSPHINATES.

I. Reactions of Organophosphinates with Three Enzymes

METHODS

Hydrolysis of 4-nitrophenyl organophosphinates by rabbit serum arylesterase (purified 15-fold by PEG-4000 precipitation and DEAE chromatography, Zimmerman and Brown, [1985]) was determined at 24° in 0.1 M MOPS pH 7.5 and 2.5 mM $CaCl_2$ by change in spectroscopic absorbance at 405 nm. Michaelis constants were calculated from Woolf plots of v versus v/[s]. Carboxylesterase inhibition was determined 2 min. after exposure to phosphinate by colorimetric measurement of hydpolytic activity remaining against 1-naphthyl acetate. (Bryson and Brown 1985).

RESULTS

Ten of 13 phosphinates were substrates for arylesterase with lower Michaelis constants than paraoxon (Table 1). Within each series of compounds, this spectrum of activity resembled that of acetylcholinesterase inhibition (Fig. 1). Reactivity of diphenyl and di-2-thienyl derivatives varied among 3 enzymes and all phosphinates tested were rapid carboxylesterase inhibitors.

TABLE I. MICHAELIS CONSTANTS AND SPECIFIC ACTIVITIES OF 4-NITROPHENYL ORGANOPHOSPHINATES WITH ARYLESTER HYDROLASE FROM RABBIT SERUM

	SUBSTITUENTS	RT[A]	K_M, MM±S.E (N)	SPECIFIC ACTIVITY μMOLES/MIN/MG
PHENYL SERIES				
I	METHYL(PHENYL)	3.85	0.0750±0.047 (4)	2.9
II	ETHYL(PHENYL)	5.05	0.285±0.048 (4)	0.65
III	ISOPROPYL(PHENYL)	6.58	B (2)	ND
IV	DIPHENYL	9.68	B (2)	ND
METHYL SERIES				
V	METHYL(2-FURYL)	3.12	0.0210±0.014 (2)	1.4
VI	METHYL(2-THIENYL)	3.68	0.0309 ± 0.0054 (4)	2.5
(I)	METHYL(PHENYL)	3.85	0.0750±0.047 (4)	2.9
VII	METHYL(1-NAPHTHYL)	7.31	0.124±0.0621 (4)	0.19
HETEROCYCLE SERIES				
(V)	METHYL(2-FURYL)	3.12	0.0210±0.014 (2)	1.4
(VI)	METHYL(2-THIENYL)	3.68	0.0309±0.0054 (4)	2.5
VIII	DI-2-THIENYL	7.67	0.0744±0.0134 (4)	0.039
HALOGEN SERIES				
IX	BIS CHLOROMETHYL	3.83	0.434±0.104 (4)	ND
X	MONOCHLOROMETHYL(PHENYL)	5.74	0.0567±0.0082 (4)	1.6
XI	DICHLOROMETHYL(PHENYL)	8.89	0.167±0.011 (4)	ND
XII	TRICHLOROMETHYL(PHENYL)	15.87	B (2)	ND
XIII	METHYL(TRIFLUOROMETHYLPHENYL)	ND	0.494±0.337 (2)	ND
XIV	PARAOXON	5.1	0.609±0.023 (17)	0.056
XV	METHYL PARAOXON	ND	ND	0.036

[A]RETENTION TIMES ON OCTASILYL BONDED COLUMN FROM BROWN AND GROTHUSEN (1983) CORRECTED FOR DEAD VOLUME.

[B]ENZYMATIC HYDROLYSIS WAS BELOW DETECTION.

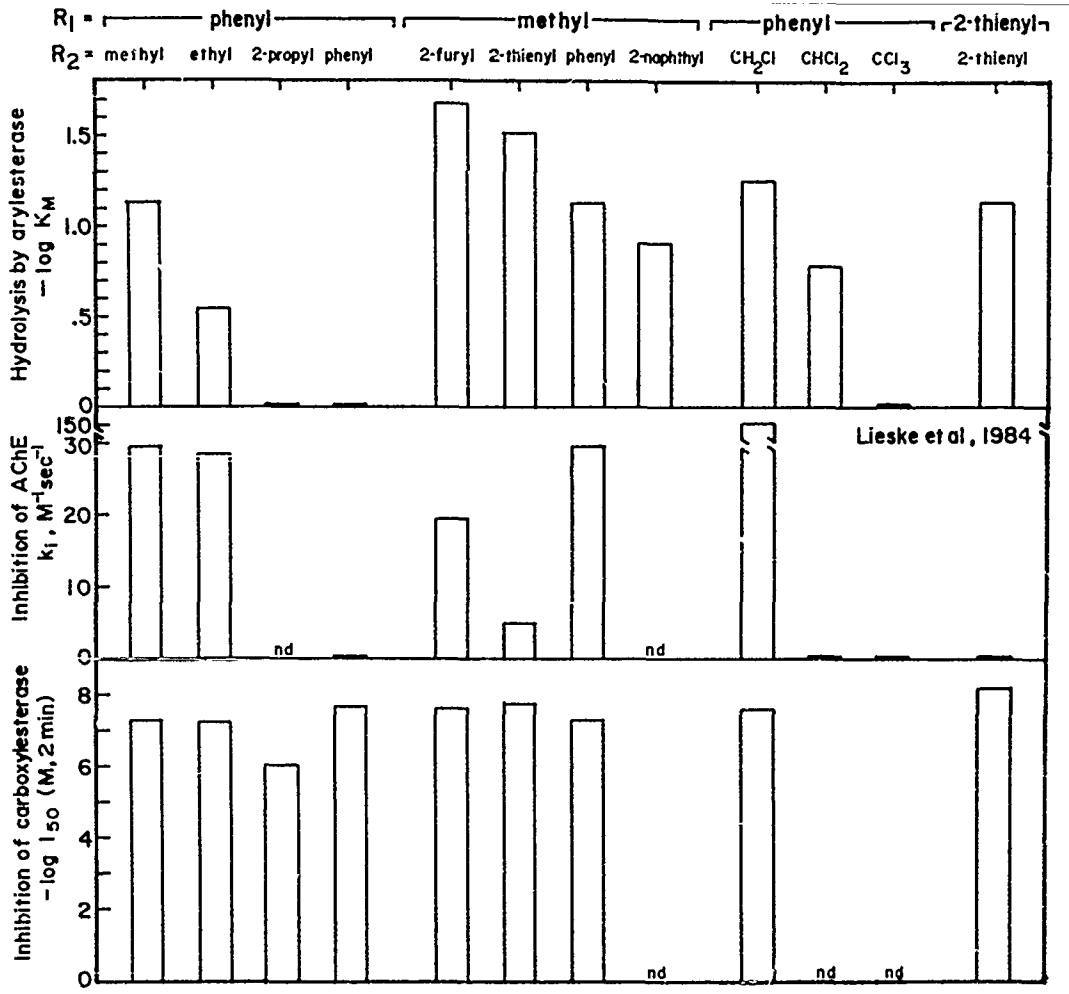

FIGURE 1. REACTION OF 4-NITROPHENYL ORGANOPHOSPHINATES WITH RABBIT SERUM ARYLESTERASE, ELECTRIC EEL ACETYLCHOLINESTERASE AND PORCINE LIVER OLIGOMERIC CARBOXYLESTERASE. PHOSPHINATES ARE INDICATED ABOVE FIGURE BY THEIR P-C BONDED SUBSTITUENTS.

II. SPONTANEOUS AND INDUCED REACTIVATION OF CARBOXYLESTERASE

METHODS

RABBIT LIVER MONOMERIC CARBOXYLESTERASE WAS INHIBITED BY 4.85×10^{-4} PHOSPHINATE FOR 2 MIN. INHIBITED CARBOXYLESTERASE WAS SEPARATED FROM INHIBITOR BY SEPHADEX CHROMATOGRAPHY. REACTIVATION AT 37° WAS DETERMINED COLORIMETRICALLY BY ACTIVITY OF ALIQUOTS AGAINST 1-NAPHTHYL BUTYRATE (BRYSON AND BROWN 1985).

RESULTS

SPONTANEOUS REACTIVATION OF 11 OF 13 PHOSPHINYLED CARBOXYLESTERASES WAS MORE RAPID THAN CARBOXYLESTERASE PHOSPHORYLATED BY PARAOXON (TABLE 2). THE SPECTRUM OF SPONTANEOUS REACTIVATION WAS SIMILAR TO THOSE OBSERVED FOR PHOSPHINYLATED HUMAN CHOLINESTERASES (FIG. 2). REACTIVATION INDUCED BY TMB-4 WAS TWICE AS RAPID AS SPONTANEOUS RECOVERY (FIG. 3).

TABLE 2. SPONTANEOUS REACTIVATION OF CARBOXYLESTER HYDROLASE FOLLOWING INHIBITION BY SUBSTITUTED 4-NITROPHENYL ORGANOPHOSPHINATES.

COMPOUND	SUBSTITUTIONS ON PHOSPHINATE	REACTIVATION, % OF CONTROL ACTIVITY ± S.E. 24 H	72 H	$\underline{K}$, MIN^{-1}*
I	METHYL(PHENYL)	65.3 ± 5.6	65.5 ± 4.8	6.7×10^{-4}
VI	METHYL(2-THIENYL)	57.6 ± 2.8	66.0 ± 2.5	5.8×10^{-4}
V	METHYL(2-FURYL)	51.9 ± 4.9	85.1 ± 2.1	3.9×10^{-4}
II	ETHYL(PHENYL)	50.8 ± 1.9	65.8 ± 2.6	4.8×10^{-4}
IX	DI-2-THIENYL	44.4 ± 8.2	84.0 ± 9.1	6.0×10^{-4}
XI	MONOCHLOROMETHYL(PHENYL)	26.5 ± 2.2	40.2 ± 14.6	-
IV	DIPHENYL	18.5 ± 3.9	39.0 ± 3.0	1.2×10^{-4}
XII	DICHLOROMETHYL(PHENYL)	14.2 ± 3.5	21.4 ± 3.5	-
X	BIS-CHLOROMETHYL	12.4 ± 4.6	13.6 ± 9.4	-
VIII	DI-2-FURYL	10.6 ± 1.3	11.4 ± 1.0	-
VII	METHYL(1-NAPHTHYL)	6.8 ± 0.4	14.7 ± 4.7	-
XIII	TRICHLOROMETHYL(PHENYL)	6.5 ± 2.0	7.5 ± 5.2	-
-	PARAOXON	5.9 ± 2.1	5.4 ± 3.6	-
III	ISOPROPYL(PHENYL)	1.5 ± 0.9	4.4 ± 1.0	9.9×10^{-6}

* REACTIVATION RATE CONSTANT BASED ON FIRST-ORDER PORTION OF REACTIVATION CURVE WHICH INCLUDED DATA THROUGH 24 H FOR I, II AND VI AND DATA THROUGH 96 H FOR III, IV, V AND IX.

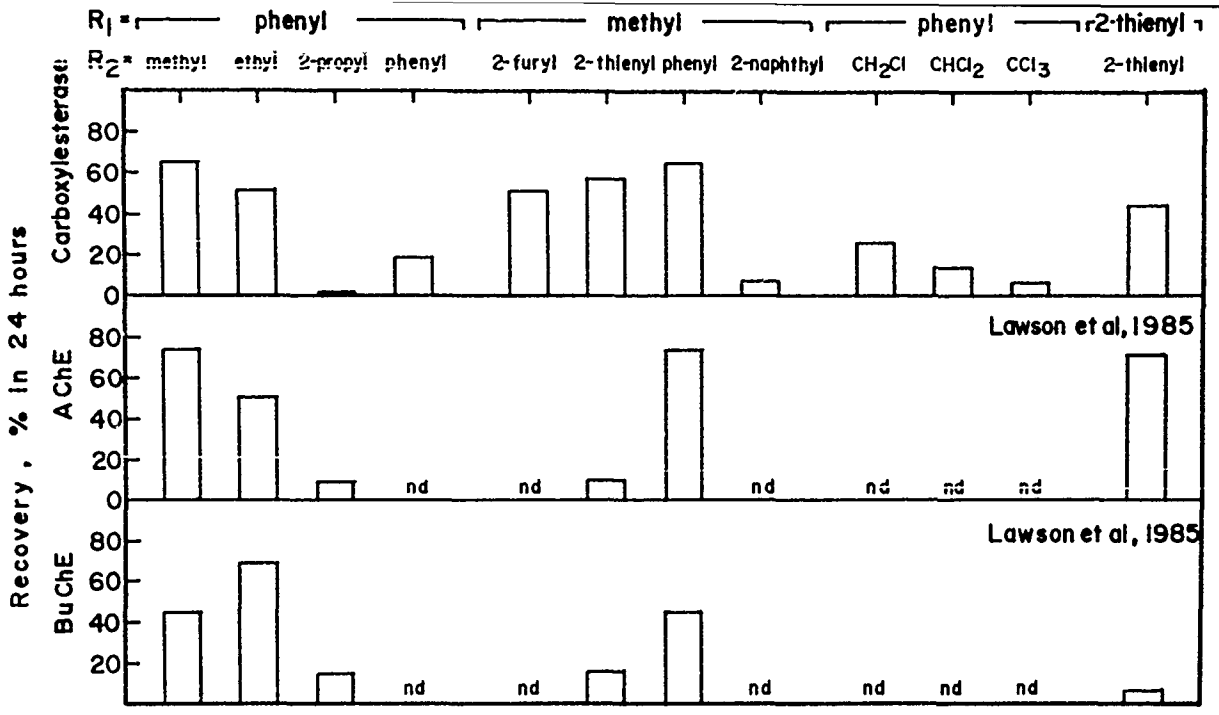

FIGURE 2. SPONTANEOUS REACTIVATION OF RABBIT LIVER MONOMERIC CARBOXYLESTERASE, HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE AND HUMAN SERUM BUTYRYLCHOLINESTERASE FOLLOWING INHIBITION BY 4-NITROPHENYL ORGANOPHOSPHINATES. PHOSPHINATES ARE INDICATED ABOVE FIGURE BY THEIR P-C BONDED SUBSTITUENTS.

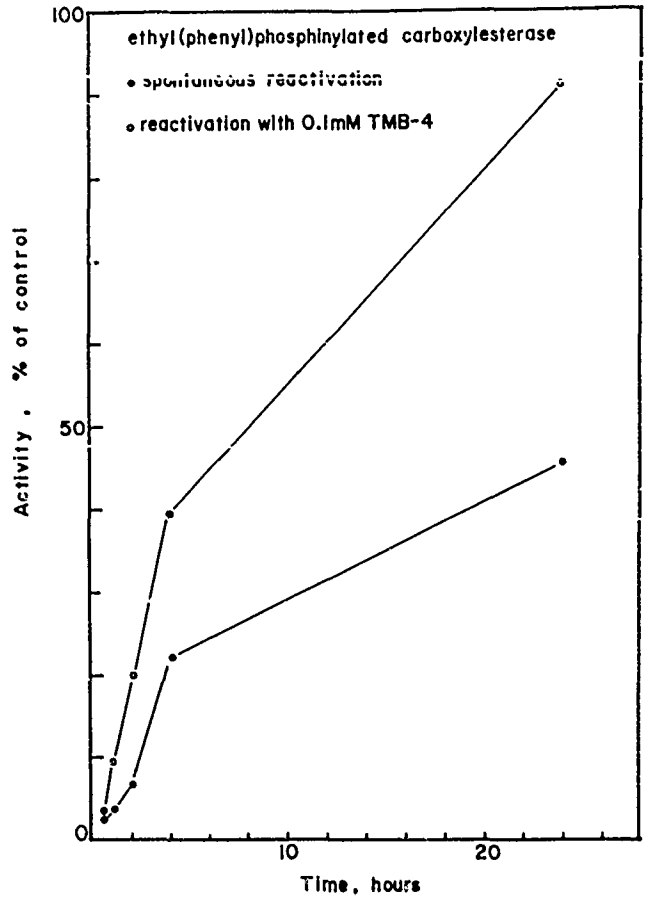

FIGURE 3. SPONTANEOUS AND TMB-4 INDUCED REACTIVATION OF CARBOXYLESTERASE PHOSPHINYLATED BY 4-NITROPHENYL ETHYL(PHENYL)PHOSPHINATE

III. Quantitative Structure - Activity Relationships

METHODS

Models of the phosphinates were constructed using a CPK kit. Each model was placed on a copy machine with the two P-C bonded substituents flat against the glass. The conformation chosen was that which gave the minimum amount of overlap between the two substituents. The silhouettes were cut out and areas determined by weighing. This procedure is similar to that described by Amoore (1965). A value of one angstrom per 1.25 cm of model length was used to convert from model size to molecular size.

Retention times for the phosphinates on octasilyl bonded silica HPLC are from Brown and Grothusen (1983) corrected for void volume. The values for octanol-water partitioning are from C. N. Lieske (personal communication).

RESULTS

There are many factors that need to be considered in any attempt to develop quantitative-structure-activity relationships for these compounds with enzymes. Three very important factors are the leaving group, hydrophobicity and molecular size. The leaving group for all of the phosphinates we are studying is the same, 4-nitrophenol. Hydrophobicity can be measured by octanol-water partitioning, or by reverse phase HPLC (Mirrlees et. al 1976; Unger et. al 1978). Reverse phase HPLC analysis is very quick and is not complicated by spontaneous hydrolysis problems. We get excellent correlation between the octanol-water partition values (Lieske, personal communication) and our reverse phase retention times for the phosphinates.

If the phosphinates are grouped into series by their common substituents (methyl, phenyl, halogen or heterocycle), there seems to be reasonable correlation with enzyme interaction and molecular size as indicated in the figures in sections I and II of this presentation. When the phosphinates are considered as one group, quantitative correlation of enzyme interaction with molecular size or retention time deteriorates.

AS A FIRST APPROXIMATION OF THE RELATIVE IMPORTANCE OF EACH OF THE TWO FACTORS, HYDROPHOBICITY AND SUBSTITUENT SIZE, WE ATTEMPTED LINEAR CORRELATIONS OF THE DATA (SECTIONS I AND II). SOME OF THE ATTEMPTED CORRELATIONS ARE SUMMARIZED BELOW (R IS THE CORRELATION COEFFICIENT OF LINEAR LEAST SQUARES).

	R	N
- LOG KM (ARYLESTERASE) VS. SIZE	- 0.18	9
- LOG KM (ARYLESTERASE) VS. RETENTION TIME	- 0.29	9
SPECIFIC ACTIVITY (ARYLESTERASE) VS. SIZE	- 0.79	10
SPECIFIC ACTIVITY (ARYLESTERASE) VS. RETENTION TIME	- 0.66	10
- LOG KI (ACETYLCHOLINESTERASE) VS. SIZE	- 0.70	10
- LOG KI (ACETYLCHOLINESTERASE) VS. RETENTION TIME	- 0.59	10
I_{50} (CARBOXYLESTERASE) VS. SIZE	- 0.097	8
I_{50} (CARBOXYLESTERASE) VS. RETENTION TIME	- 0.039	8
RECOVERY (CARBOXYLESTERASE) VS. SIZE	- 0.53	13
RECOVERY (CARBOXYLESTERASE) VS. RETENTION TIME	- 0.55	13

IT IS OBVIOUS FROM THESE RESULTS THAT NEITHER SIZE NOR HYDROPHOBICITY ALONE CAN BE USED TO ACCURATELY DEFINE QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS. A COMBINATION OF FACTORS MUST BE CONSIDERED. NON-LINEAR RELATIONSHIPS MUST ALSO BE CONSIDERED.

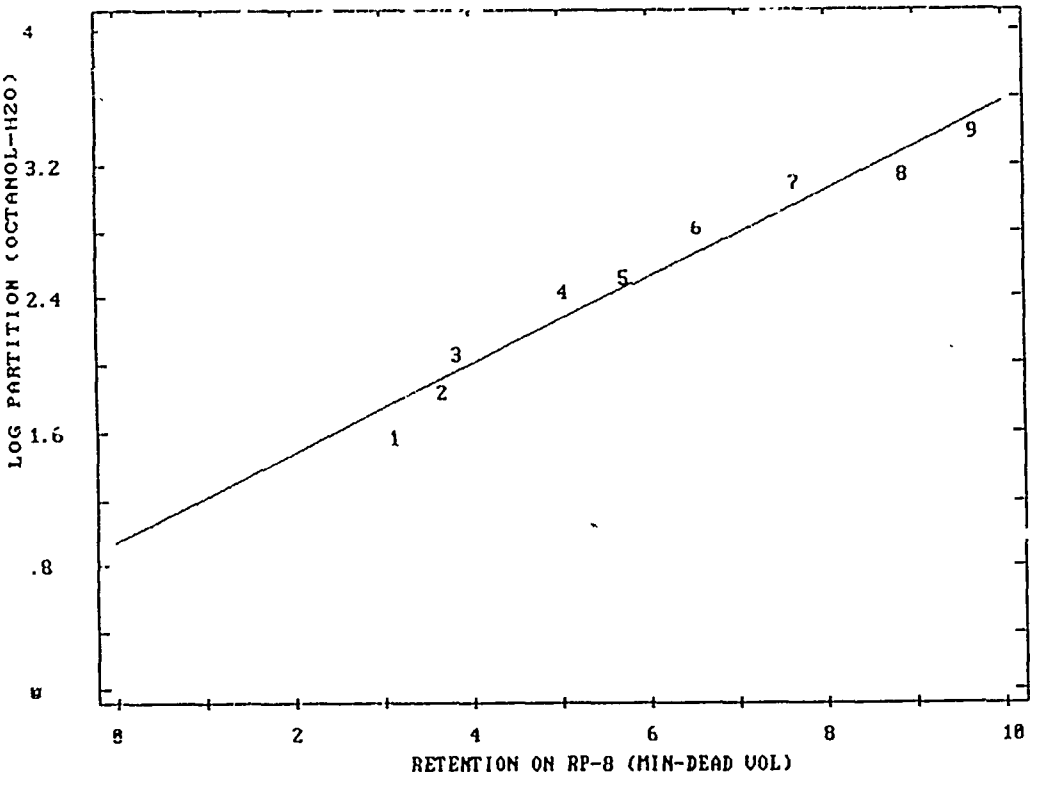

FIGURE 4. CORRELATION OF OCTANOL-WATER PARTITION VALUES WITH RETENTION TIME ON OCTASILYL BONDED HPLC COLUMN FOR THE 4-NITROPHENYL ORGANOPHOSPHINATES. OCTANOL-WATER PARTITION VALUES ARE FROM C. N. LIESKE (PERSONAL COMMUNICATION). RETENTION TIMES ARE FROM BROWN AND GROTHUSEN (1983).

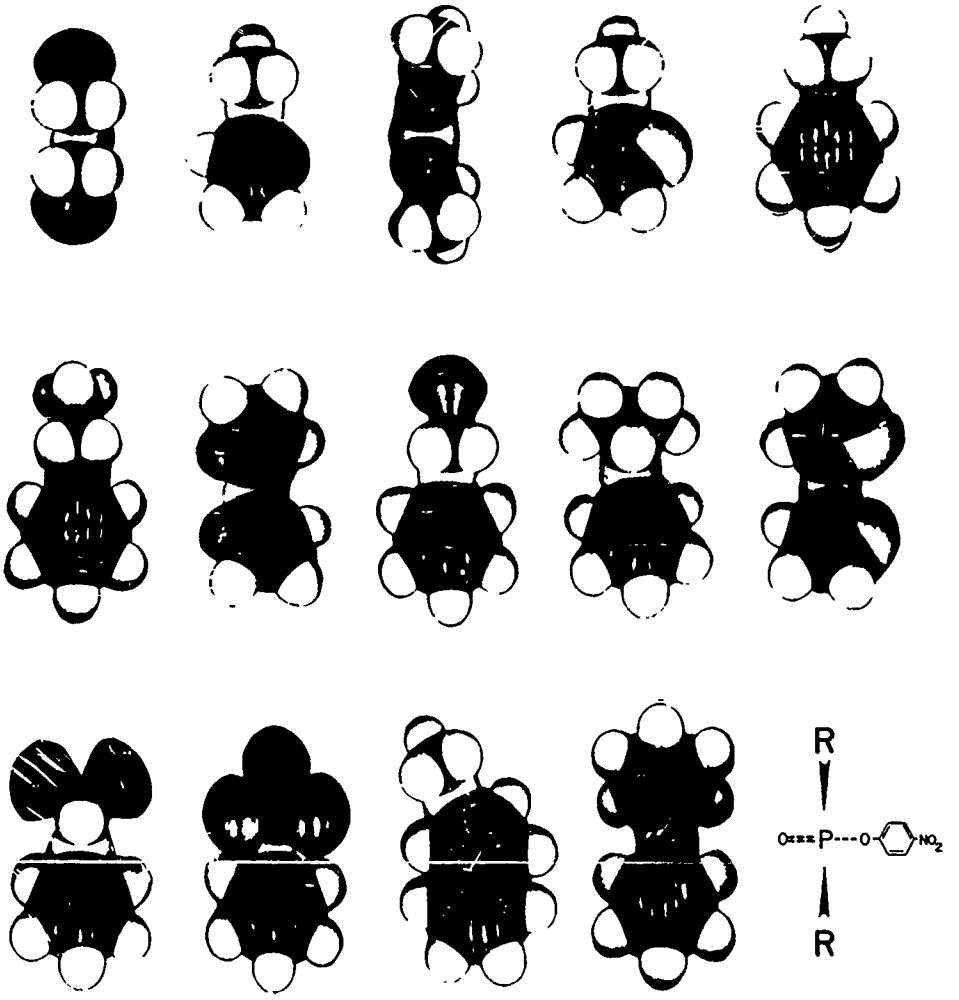

FIGURE 5. SILHOUETTES OF THE PHOSPHORUS-CARBON BONDED SUBSTITUENTS OF THE 4-NITROPHENYL ORGANOPHOSPHINATES. THE SILHOUETTES ARE ARRANGED FROM SMALLEST (UPPER LEFT BIS-CHLOROMETHYL) TO LARGEST (LOWER RIGHT DIPHENYL).

IV. Stereochemistry of Enzymatic Hydrolysis of Phosphinates

METHODS

In the studies with paraoxonase, a small amount of enzyme (20µL) was added to 1 mL of 0.1M MOPS buffer (+ 2.4 mM $CaCl_2$, pH 7.5) along with the phosphinate. The phosphinate concentrations in the reaction mixture were 6-8 mM. Aliquots were removed and extracted 3 times in acidified EtOAc versus H_2O. The EtOAc was evaporated to dryness and the sample taken up in 100µL of acetonitrile. A 10µL sample is injected on a Regis Pirkle covalent D. or L-phenylglycine chiral stationary phase HPLC column.

The studies of phosphinylation of trypsin or chymotrypsin use a 1:1 molar ratio of phosphinate to protein. The concentration of enzyme and phosphinate were usually 0.34 mM in 20 mL of 0.1 M MOPS buffer. The reaction mixture was maintained at 37°C and 10 mL aliquots were removed. Protein was removed by addition of 1:1 acetone/acetonitrile, followed by centrifugation. Phosphinate was extracted using methylene chloride versus H_2O. The methylene chloride fractions were taken to dryness and resuspended in a known amount of acetonitrile. Samples were analyzed on the Pirkle CSP column (Figures 7 and 8).

RESULTS

There appeared to be a preferential hydrolysis of the longer retained enantiomer of 4-nitrophenyl ethyl(phenyl)phosphinate by rabbit serum paraoxonase as analysed on the Pirkle D-phenylglycine column. The longer retained enantiomers of 4-nitrophenyl ethyl(phenyl) and 4-nitrophenyl isopropyl(phenyl)phosphinates preferentially phosphinylated the serine proteases trysin and chymotrypsin as evidenced by the dissappearence of the longer retained peaks (Figures 9, 10 and 11).

We are currently studying the inhibition of various enzymes, including acetylcholinesterase, by purified enantiomers of EPP and IPP. The purified enantiomers are obtained by repetitive trapping of peaks from the chiral stationary phase columns.

FIGURE 6. CPK MODELS OF THE TWO ENANTIOMERS OF 4-NITROPHENYL ETHYL(PHENYL)-PHOSPHINATE (EPP)

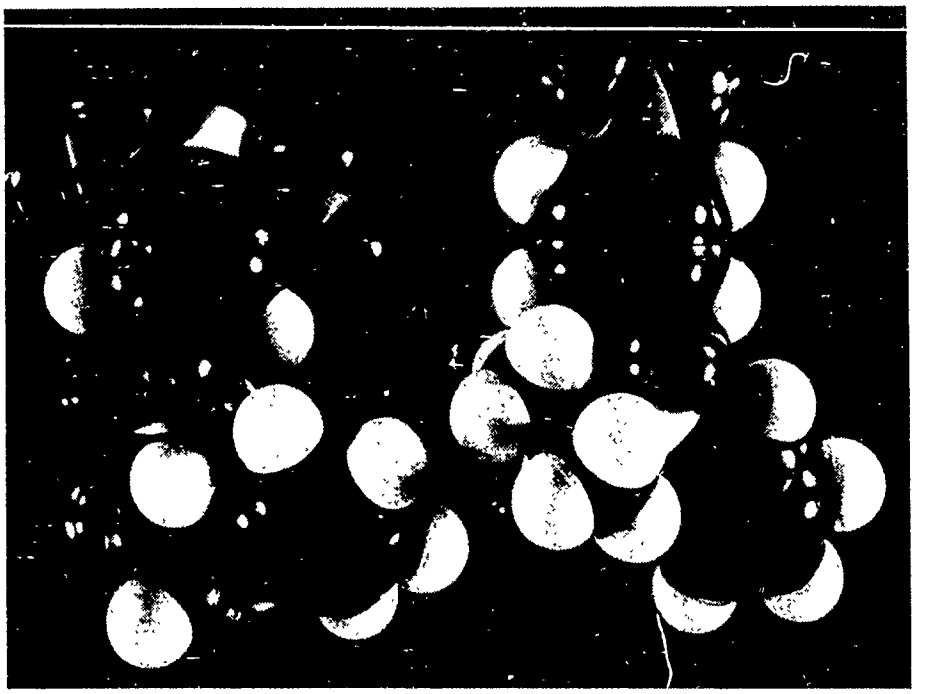

FIGURE 7. THE LEFT SIDE IS A MODEL OF THE D-PHENYLGLYCINE CHIRAL STATIONARY PHASE ((R)-N-3,5-DINITROBENZOYLPHENYLGLYCINE). ON THE RIGHT IS A MODEL OF ONE OF THE ENANTIOMERS OF 4-NITROPHENYL ISOPROPYL(PHENYL)-PHOSPHINATE (IPP).

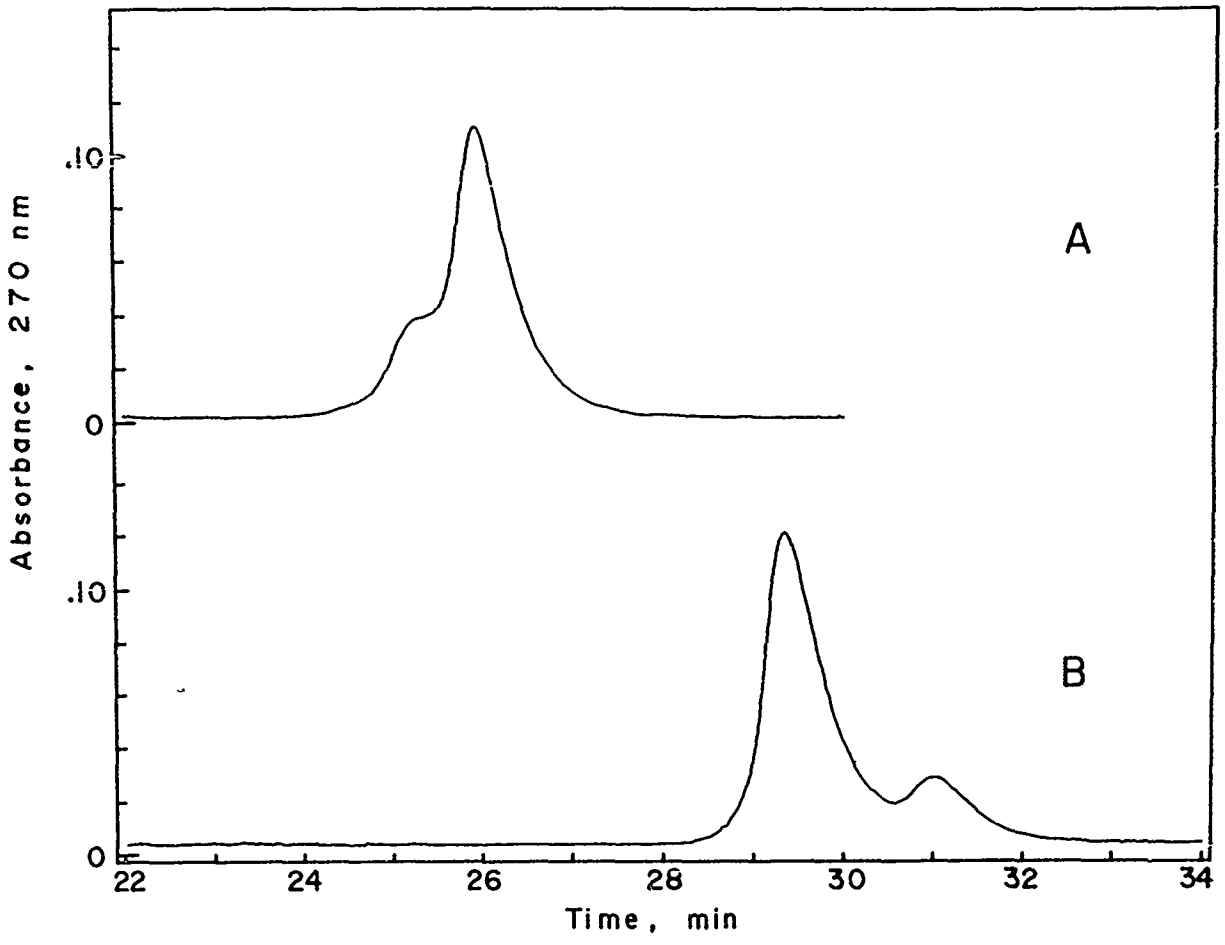

FIGURE 8. COMPARISON D- AND L-PHENYLGLYCINE CHIRAL STATIONARY PHASE (CSP) COLUMNS

A. SAMPLE OF PARTIALLY PURIFIED ENANTIOMER OF 4-NITROPHENYL ISOPROPYL-(PHENYL)PHOSPHINATE (IPP) CHROMATOGRAPHED ON D-PHENYLGLYCINE DERIVATIVE PIRKLE CSP COLUMN

B. SAME SAMPLE CHROMATOGRAPHED ON L-PHENYLGLYCINE DERIVATIVE PIRKLE CSP COLUMN

BOTH COLUMNS ARE REGIS COVALENT CSP COLUMNS. RUNNING CONDITIONS IN BOTH CASES: 7% 2-PROPANOL IN HEXANE PUMPED 1 mL/MIN. AT 18°C.

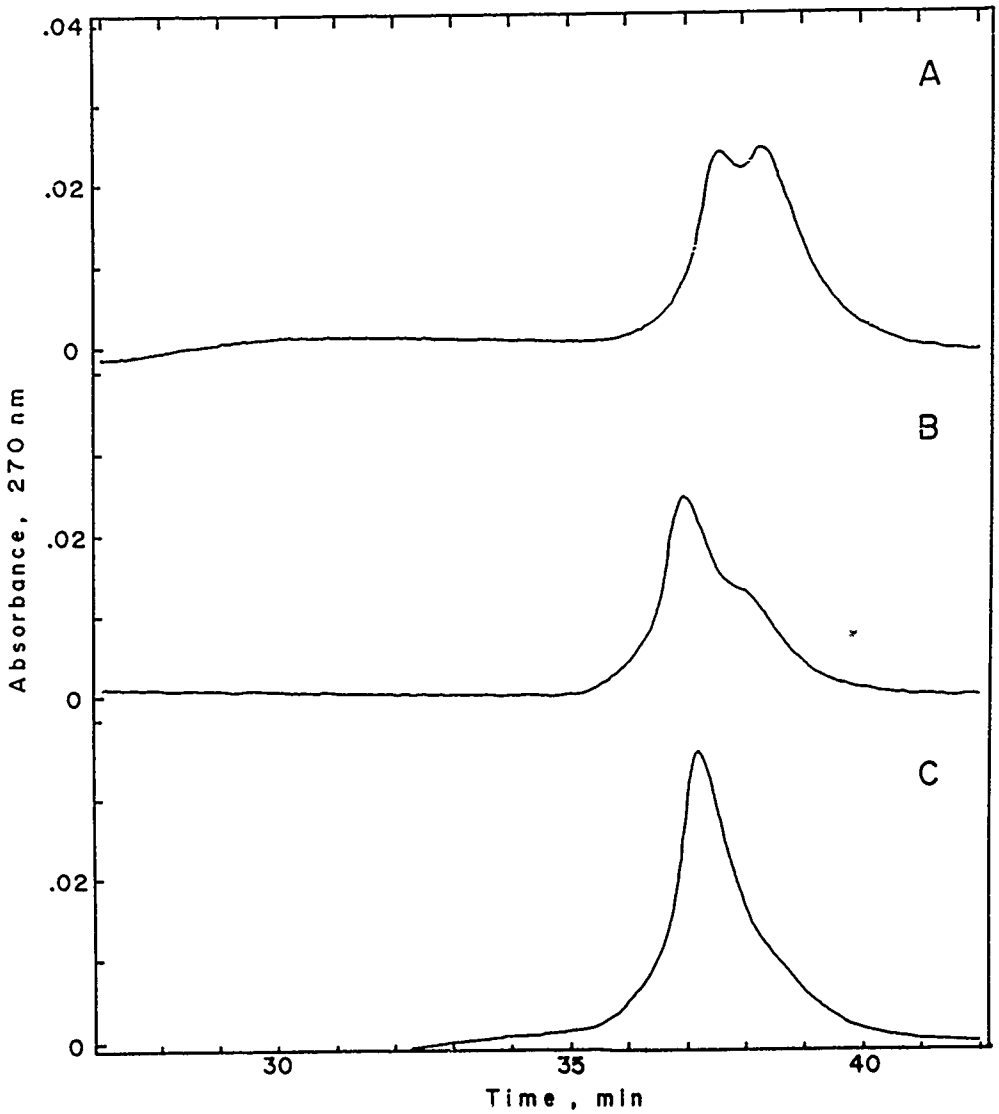

FIGURE 9. STEREOSELECTIVE HYDROLYSIS OF 4-NITROPHENYL ETHYL(PHENYL)PHOSPHINATE (EPP) BY ARYLESTER HYDROLASE FROM RABBIT SERUM

A. EPP IN MOPS BUFFER FOR 23 HOURS AT 24°C (SPONTANEOUS HYDROLYSIS CONTROL)

B. EPP (7.4 MM) AND ARYLESTER HYDROLASE (0.04 MG/ML) AFTER 60 MIN. AT 24°C

C. EPP AND ARYLESTER HYDROLASE AFTER 150 MIN. AT 24°C

CHROMATOGRAMS OF EPP ON PIRKLE D-PHENYLGLYCINE CSP COLUMN AFTER REMOVAL OF PROTEIN FROM SAMPLES

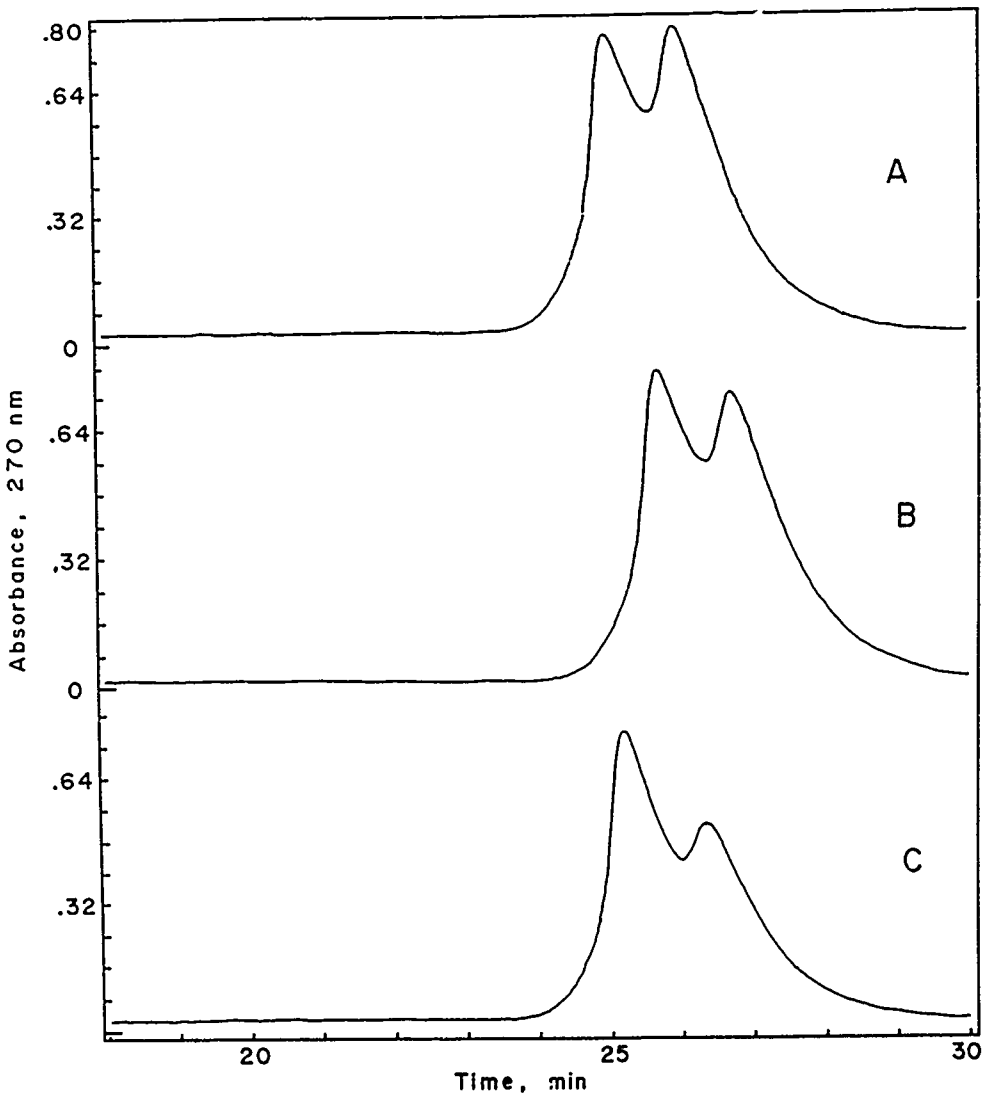

FIGURE 10. STEREOSELECTIVE PHOSPHINYLATION OF TRYPSIN BY 4-NITROPHENYL ISOPROPYL(PHENYL)PHOSPHINATE (IPP)

A. IPP (0.34 mM) INCUBATED AT 37°C FOR 40 MIN. IN MOPS BUFFER

B. IPP (0.34 mM) AND TRYPSIN (0.34 mM) INCUBATED AT 37°C FOR 6 MIN.

C. IPP AND TRYPSIN INCUBATED FOR 40 MIN.

CHROMATOGRAMS OF IPP ON PIRKLE D-PHENYLGLYCINE CSP COLUMN AFTER EXTRACTION OF PHOSPHINATE FROM REACTION MIXTURE

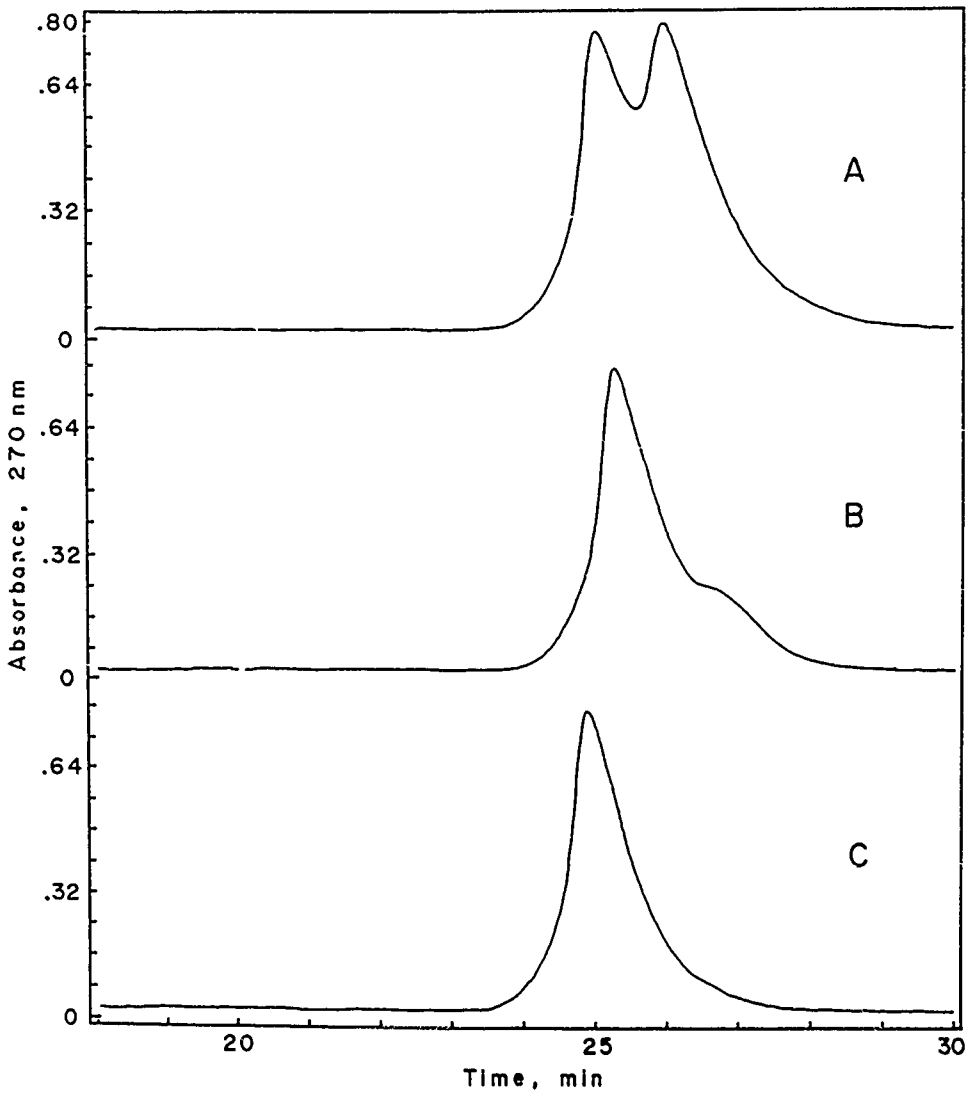

FIGURE 11. STEREOSELECTIVE PHOSPHINYLATION OF α-CHYMOTRYPSIN BY 4-NITROPHENYL ISOPROPYL(PHENYL)PHOSPHINATE (IPP)

A. IPP (0.34 MM) INCUBATED AT 37°C FOR 40 MIN. IN MOPS BUFFER

B. IPP (0.34 MM) AND α-CHYMOTRYPSIN (0.34 MM) INCUBATED AT 37°C FOR 6 MIN.

C. IPP AND α-CHYMOTRYPSIN INCUBATED FOR 40 MIN.

CHROMATOGRAMS OF IPP ON PIRKLE D-PHENYLGLYCINE CSP COLUMN AFTER EXTRACTION OF PHOSPHINATE FROM REACTION MIXTURE

V. References

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SYNTHESIS OF A SOMAN ANALOG AS A HAPTEN FOR ANTIBODY PRODUCTION

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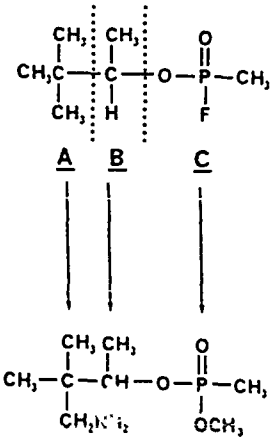
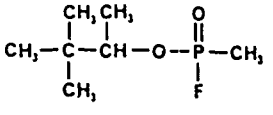
STRUCTURAL REQUIREMENTS FOR SOMAN HAPTEN

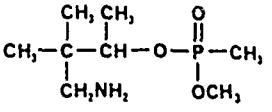


A. THE AMINOPHOSPHONATE IS ISOSTERIC WITH SOMAN
B. THE HIGHLY REACTIVE P-F BOND IS REPLACED BY P-OCH_3
C. IT MAY BE CONJUGATED TO A PROTEIN VIA SUCCINYLATION
D. IT IS STABLE TO THE CONJUGATION AND ANTIBODY PRODUCTION PROCEDURE.

SYNTHESIS OF A SOMAN ANALOG AS A HAPTEN FOR ANTIBODY PRODUCTION

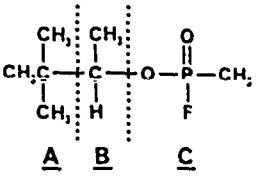


SOMAN

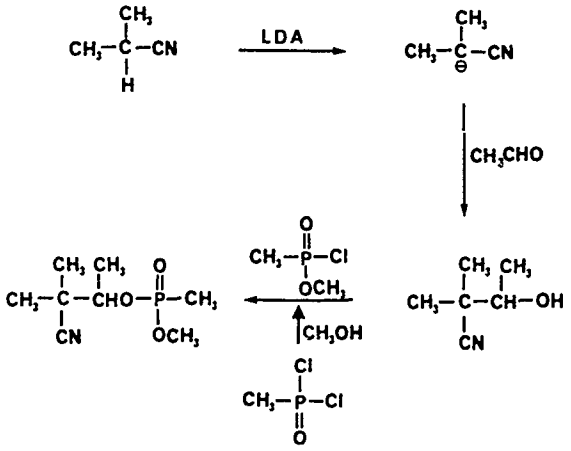


SOMAN ANALOG SUITABLE FOR CONJUGATION TO A PROTEIN AND EVENTUAL PRODUCTION OF ANTIBODIES

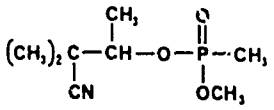
THE SOMAN MOLECULE CONSISTS OF THREE MAIN SECTIONS. THE TERT-BUTYL PART (A), THE CH_3CH- PART (B) AND THE PHOSPHONATE ESTER PART (C)



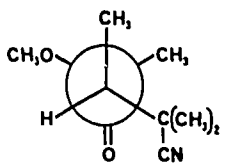
SYNTHESIS

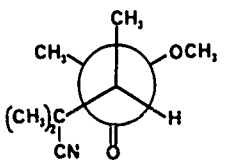


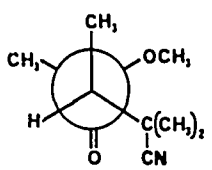
STEREOCHEMISTRY OF

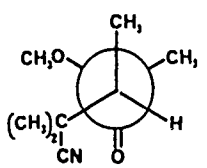


THIS MOLECULE CONTAINS TWO CHIRAL CENTERS AND CAN EXIST IN 4 STEREOISOMERIC FORMS.

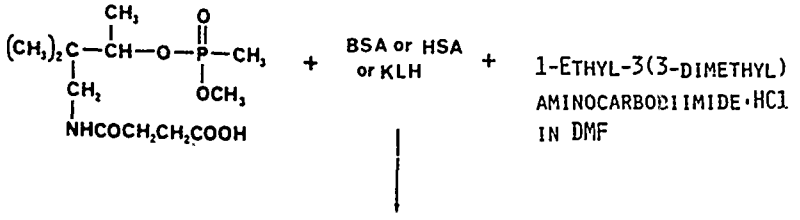








CONJUGATION OF SUCCINAMIDO DERIVATIVE TO PROTEINS

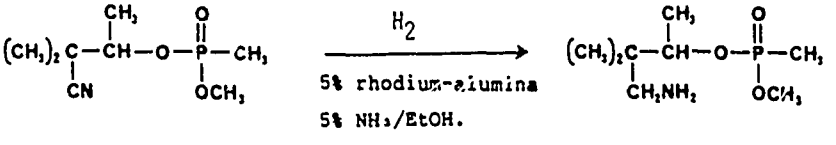


METHOD F. A. FITZPATRICK AND G. L. BUNDY, PROC. NATL. ACAD. SCI USA 75, 2689 (1979)

PROCEDURE 10MG OF AMINOPHOSPHONATE SUCCINAMIDE IN 1.0 ML DIMETHYLFORMAMIDE, BORINE SERUM ALBUMIN (20MG) AND 1-ETHYL-3(3-DIMETHYL)AMINOPROPYLCARBODIIMIDE HCl (10MG) WERE MIXED IN 6.0ML OF DISTILLED WATER. THE pH WAS MAINTAINED BETWEEN 5.4 AND 5.6 FOR 1HR AT 25°. FOR THE REACTION OF THE SUCCINAMIDE WITH KEYHOLE LIMPET HEMOCYANIN THE pH WAS MAINTAINED AT 7.0. CONJUGATES WERE DIALYZED AGAINST 4 LITERS OF WATER FOR 24HR AND THEN LYOPHILIZED.

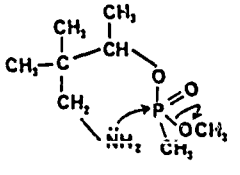
THE EPITOPE DENSITY WAS DETERMINED BY USING ^{14}C-SUCCINIC ANHYDRIDE IN THE SUCCINYLATION STEP AND DETERMINING THE RADIOACTIVITY IN THE CONJUGATE.

REDUCTION CN TO CH_2NH_2



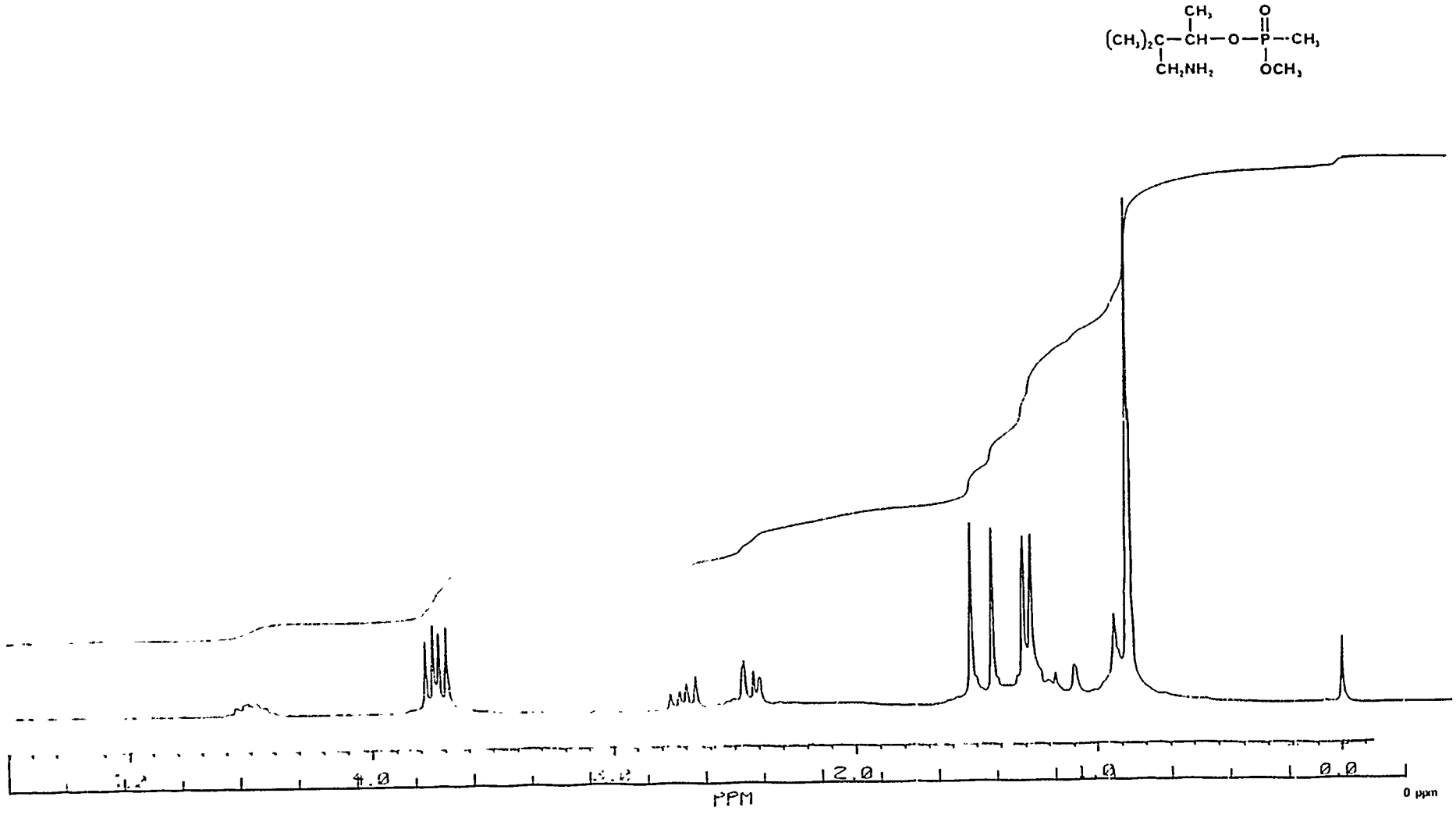
1. $-C\equiv N \longrightarrow -CH=NH \longrightarrow$ NO BIMOLECULAR CONDENSATION

2. NO INTRAMOLECULAR CYCLIZATION

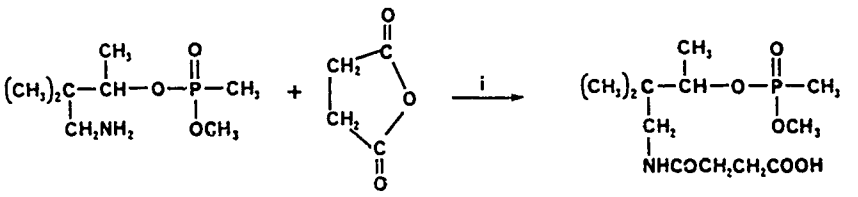


NMR OF PRODUCT

CH3 CH3 O
CH3—C—CH—O—P—CH3
CH2NH2 OCH3
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0 ppm
PPM


4.0
2.0
1.0
0.0
PPM
0 ppm

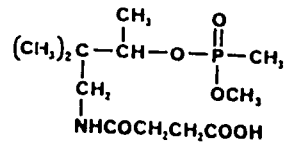
SUCCINYLATION OF AMINOPHOSPHONATE

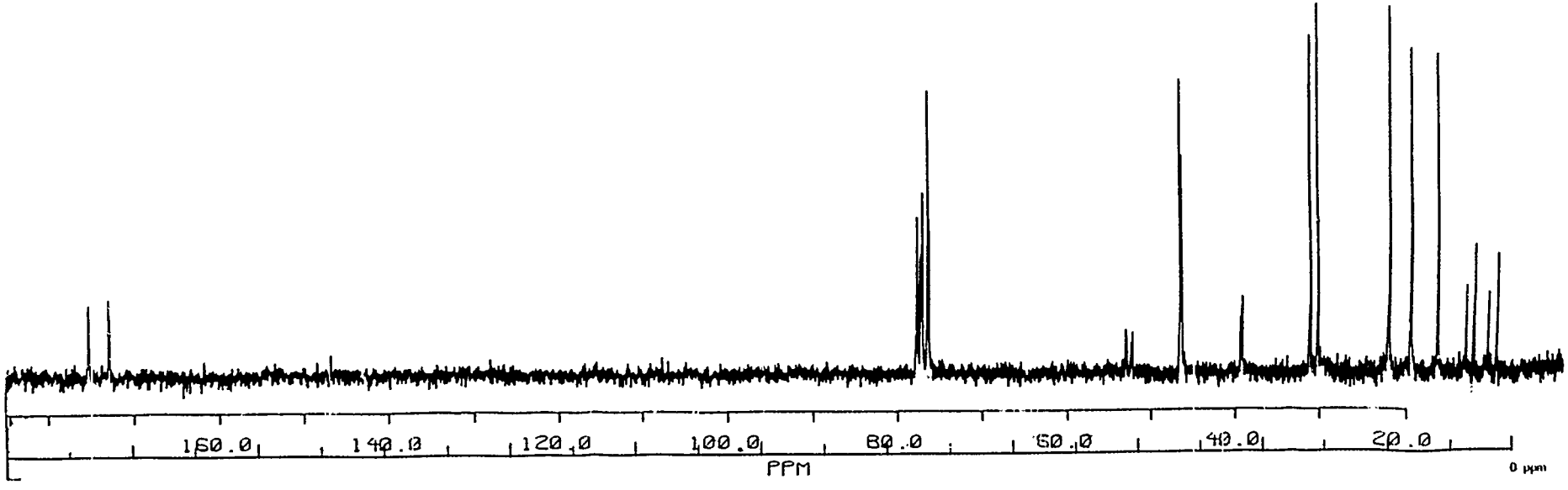


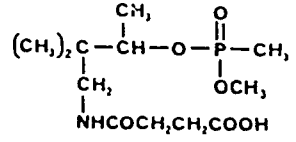
i = Et_2O-THF; reflux OVN

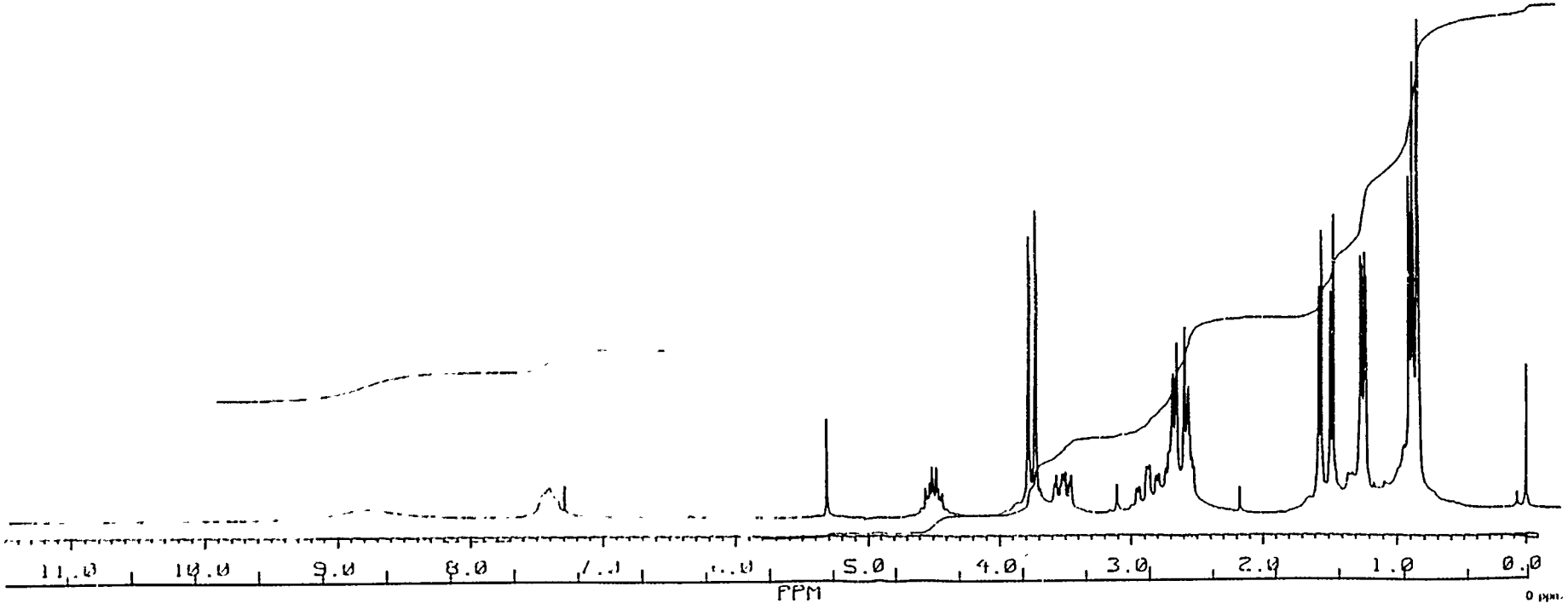
NMR

M.S.


CH3
O
(CH3)2C—CH—O—P—CH3
CH2
OCH3
NHCOCH2CH2COOH


160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
PPM
0 ppm


CH,
O
(CH,),C—CH—O—P—CH,
CH,
OCH,
NHCOCH,CH,COOH


11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM
0 ppm

SUSCEPTIBILITY OF DIFFERENT MOLECULAR WEIGHT FORMS OF ACETYLCHOLINESTERASE IN RAT BRAIN TO IN VIVO INHIBITION BY SOMAN

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ABSTRACT

The toxicity of the organophosphorus poison, soman, is attributable to its irreversible inhibition of the enzyme acetylcholinesterase (AChE). We have previously reported that the distribution of the 10S and 4S molecular weight forms of AChE varies with different brain areas (Fed. Proc., 42: 2024, 1983). Our current efforts were directed toward determining whether these different molecular weight forms exhibit selective susceptibilities to in vivo inhibition by soman. Male Sprague Dawley rats, 200 - 220 grams, were injected subcutaneously with 80, 90, or 100 μg/kg of soman. Twenty-four hours later, the brain stem and cerebral cortex were removed from the rats and homogenized in 9X (wt/v) physiologic saline containing 1% Triton X-100 and 0.02 M $MgCl_2$. The homogenates were centrifuged at 14,000 x g for 20 minutes and the supernants subsequently subjected to sucrose density gradient separation. In both brain stem and cereberal cortex there was a dose-related decrease of total AChE activity. In treated animals the ratios of uninhibited 10S to 4S forms decreased from 3:1 to 1:1 to 0.6:1 to 0.4:1 in brain stem and from 4:1 to 3.2:1 to 1.3:1 to 0.9:1 in the cerebral cortex at soman dose levels of 0, 80, 90, and 100 μg/kg respectively with the onset of the decrease occuring in the brain stem at 80 μg/kg and at 90μg/kg in the cerebral cortex. We conclude that the susceptibility to soman inhibition of the two molecular weight forms of AChE is dependent on their location in the brain areas studied and on the soman dose level.

INTRODUCTION

The toxicity of soman is attributable to its irreversible inhibition of acetylcholinesterase (AChE). We previously reported (Fed. Proc., 42: 2024, 1983), that AChE in the brain of rats existed in two different molecular weight forms, 10S and 4S, the distribution of which varied with different brain areas.

PURPOSE

In the current study we have undertaken to examine:

1. Whether these different molecular weight forms exhibit selective susceptibilities to in vivo inhibition by soman.
2. Whether any observed susceptibility is dependent upon the brain area location of these different molecular weight forms.

METHODS

Male Sprague Dawley rats, 200 - 220 grams, were injected subcutaneously on the dorsal surface with soman doses of 80, 90, or 100 μg/kg. Twenty-four hours after injection the animals were sacrificed and the entire whole brain removed. The brain was sectioned into four parts: brain stem, cerebral cortex (containing the striatum), mid brain and cerebellum. The parts were immediately frozen in liquid nitrogen and stored at $-70°C$ until needed. In the current study, brain stem and cerebral cortex were homogenized with 9 times (wt/v) physiological saline containing 1% Triton X-100 and 20mM $MgCl_2$. The 10S and 4S molecular weight forms of AChE were characterized in a 5 - 20% sucrose density gradient. Catalase (11.3S) and alkaline phosphatase (6.1S) were used as internal density markers.

AChE activity was measured by a modification of the Ellman assay. Using a 96 well, flat bottom, microtiter plate, 30 μl of each fraction was placed into a separate well. Two hundred microliters of a solution containing 10 - 3 M DTNB and 10 - 3 M acetylthiocholine in 0.1 M phosphate buffer, pH 7.0, was pipetted into each well and the change in absorbance at 414 nm was recorded as a function of time. This allowed for the analysis of AChE activity in all the fractions at essentially the same time. The ratio of the area under the curve for each molecular weight form was determined for each brain area at each dose studied.

TABLE 1.

PERCENT COMPOSITION OF REGIONAL RAT BRAIN AChE

BRAIN AREAS	SEDIMENTATION VALUES 10.3S	6.0S	4.6S
CEREBRAL CORTEX	82%		18%
MIDBRAIN	80%		14%
BRAIN STEM	70%	15%	15%
CEREBELLUM	50%		50%

The 10.3S form predominated in the cerebral cortex, midbrain, and brainstem, whereas an equal ratio of the 10.3S and 4.6S forms was found in the cerebellum. A small fraction (15%) of a 6S form was also present in the brainstem.

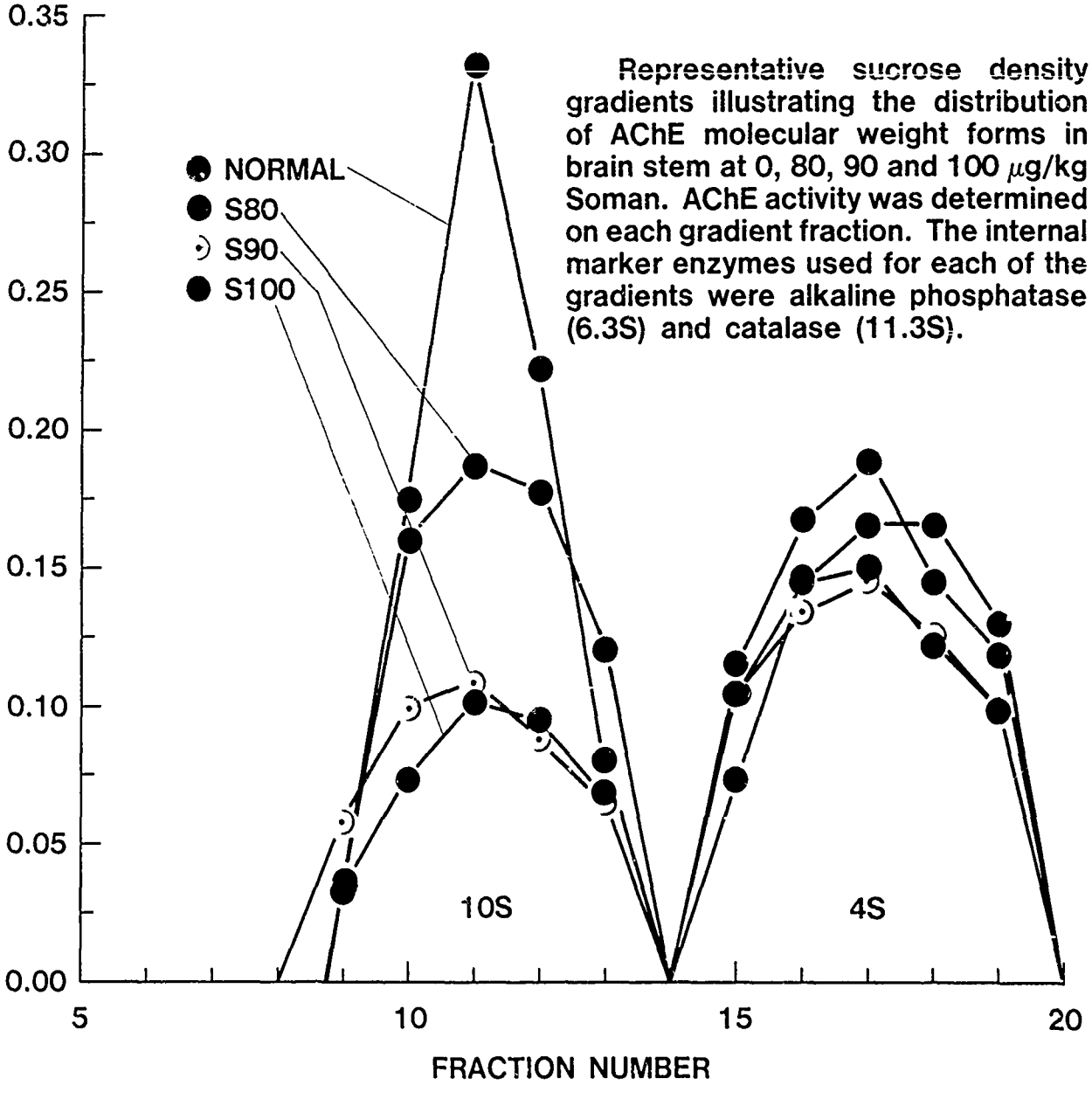
TABLE 2.

PERCENT COMPOSITION OF RAT BRAIN AREA AChE FROM SOMAN TREATED ANIMALS

	80 μg/kg SOMAN		90 μg/kg SOMAN		100 μg/kg SOMAN	
	10S	4S	10S	4S	10S	4S
CEREBRAL CORTEX	73.8	26.2	55.4	44.6	47.8	52.2
BRAIN STEM	50.0	50.0	37.6	62.4	28.0	72.0

FIGURE 1.

EFFECT OF DIFFERENT SOMAN CONCENTRATIONS ON THE ACTIVITY OF THE 10S AND 4S MOLECULAR WEIGHT FORMS OF ACETYLCHOLINESTERASE

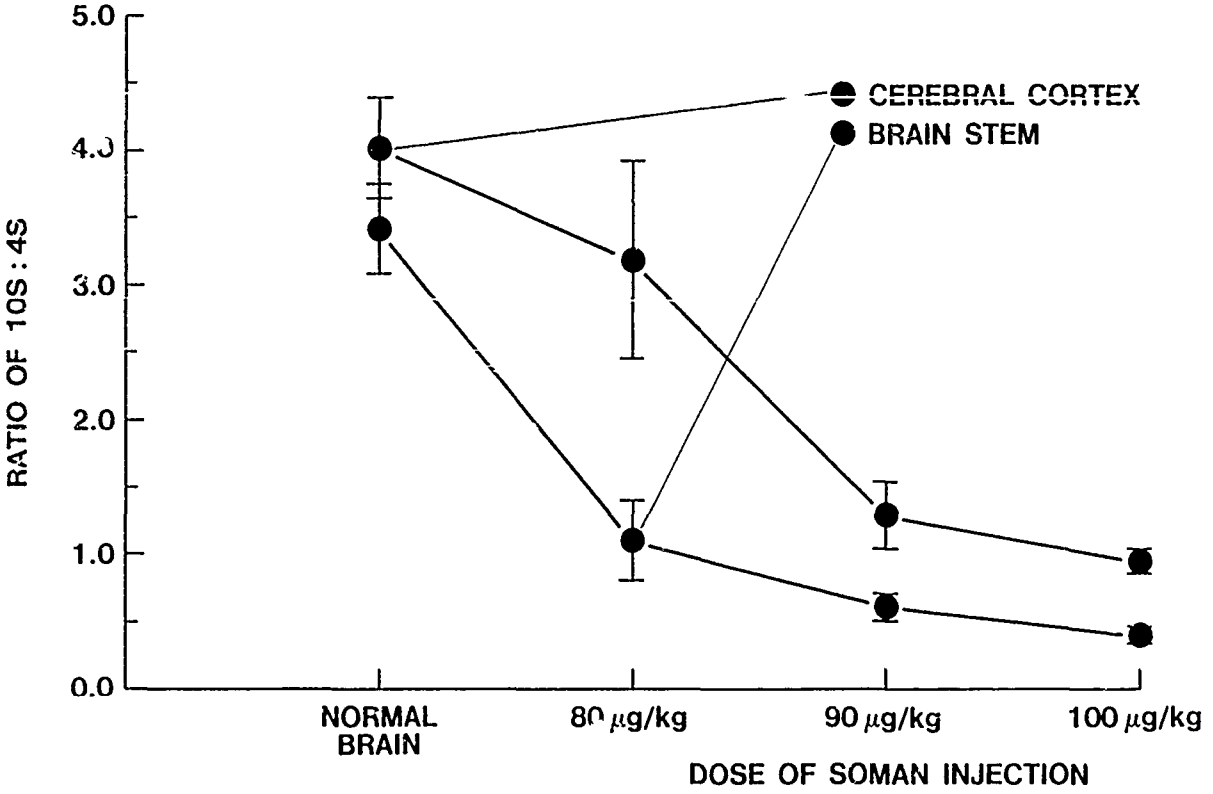


Representative sucrose density gradients illustrating the distribution of AChE molecular weight forms in brain stem at 0, 80, 90 and 100 μg/kg Soman. AChE activity was determined on each gradient fraction. The internal marker enzymes used for each of the gradients were alkaline phosphatase (6.3S) and catalase (11.3S).

A-623

FIGURE 2.

RATIO OF 10S : 4S AChE MOLECULAR WEIGHT FORMS vs SOMAN DOSE GIVEN



The ratio of 10S:4S AChE molecular weight forms in brain stem and cerebral cortex at soman concentrations of 0, 80, 90 and 100 μg/kg. The largest change in the ratio occurs at 80 μg/kg in brain stem and at 90 μg/kg in cerebral cortex.

CONCLUSIONS

1. Total AChE decreases with an increasing dose of soman in brain stem and cerebral cortex.
2. The 10S to 4S ratio decreases from 4:1 to 0.9:1 in the cerebral cortex, with the onset occuring at 90 μg/kg.
3. The 10S to 4S ratio decreases from 3.4:1 to 0.4:1 in the brain stem with the onset occuring at 80 μg/kg.
4. The change in 10S:4S ratio appears to be due to the inhibition of the 10S form, while the activity of the 4S form remains constant.

PROPHYLAXIS AGAINST NERVE AGENTS

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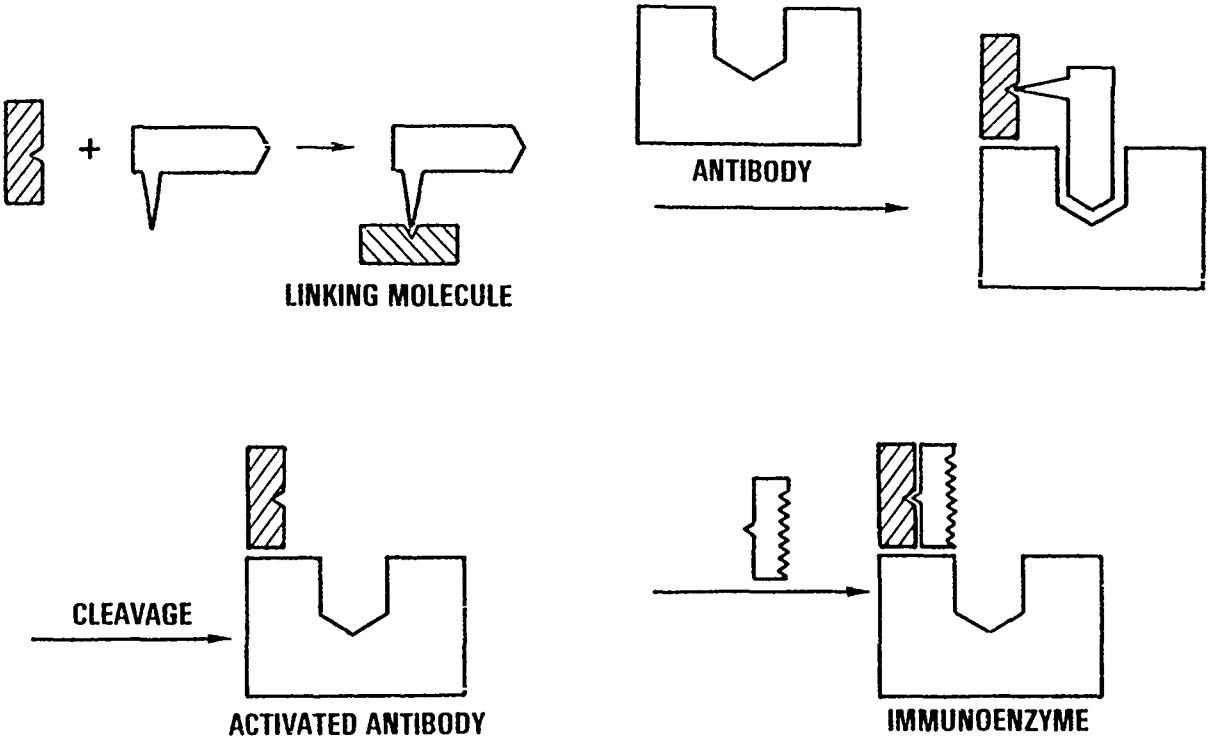
ABSTRACT

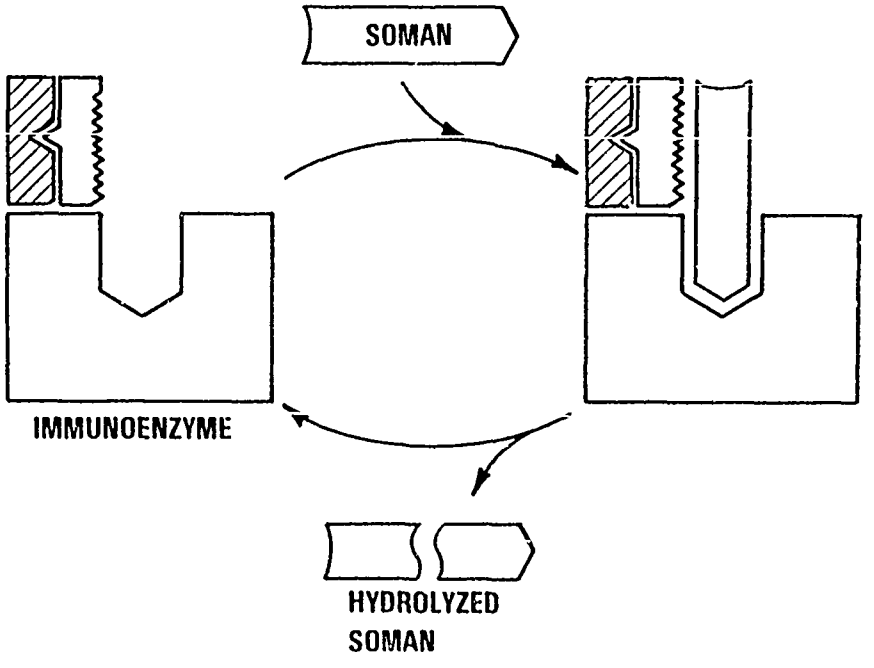
The goal of this research program is to develop a biocompatible enzyme-like catalyst for the rapid and specific deactivation of systemically sorbed nerve agents. We plan to introduce catalytic groups (thiol and imidazole groups) adjacent to the binding site of a monoclonal antibody directed against soman. In this way, we hope to construct an immunoenzyme that will catalyze the hydrolysis of soman after it is absorbed into the body but before it inhibits acetylcholinesterase. This immunoenzyme should be useful in protecting the soldier from the toxic effects of soman when administered intravenously prior to exposure.

Our first objective for preparation of the immunoenzyme is to chemically synthesize the linking molecule used to position the catalytic groups near the binding site of the antibody. Then, the catalytic moiety must be synthesized. This compound must as a minimum contain an imidazole group, a thiol group, and a group that can be used for covalent attachment to the activated antibody. (See accompanying synthetic scheme.) Understandably, the need for so many functionalities within the confines of a necessarily small molecule has made the synthesis of the catalytic moiety difficult in unanticipated ways. Our progress in the synthesis of both the linking molecule and the catalytic moiety is shown here.

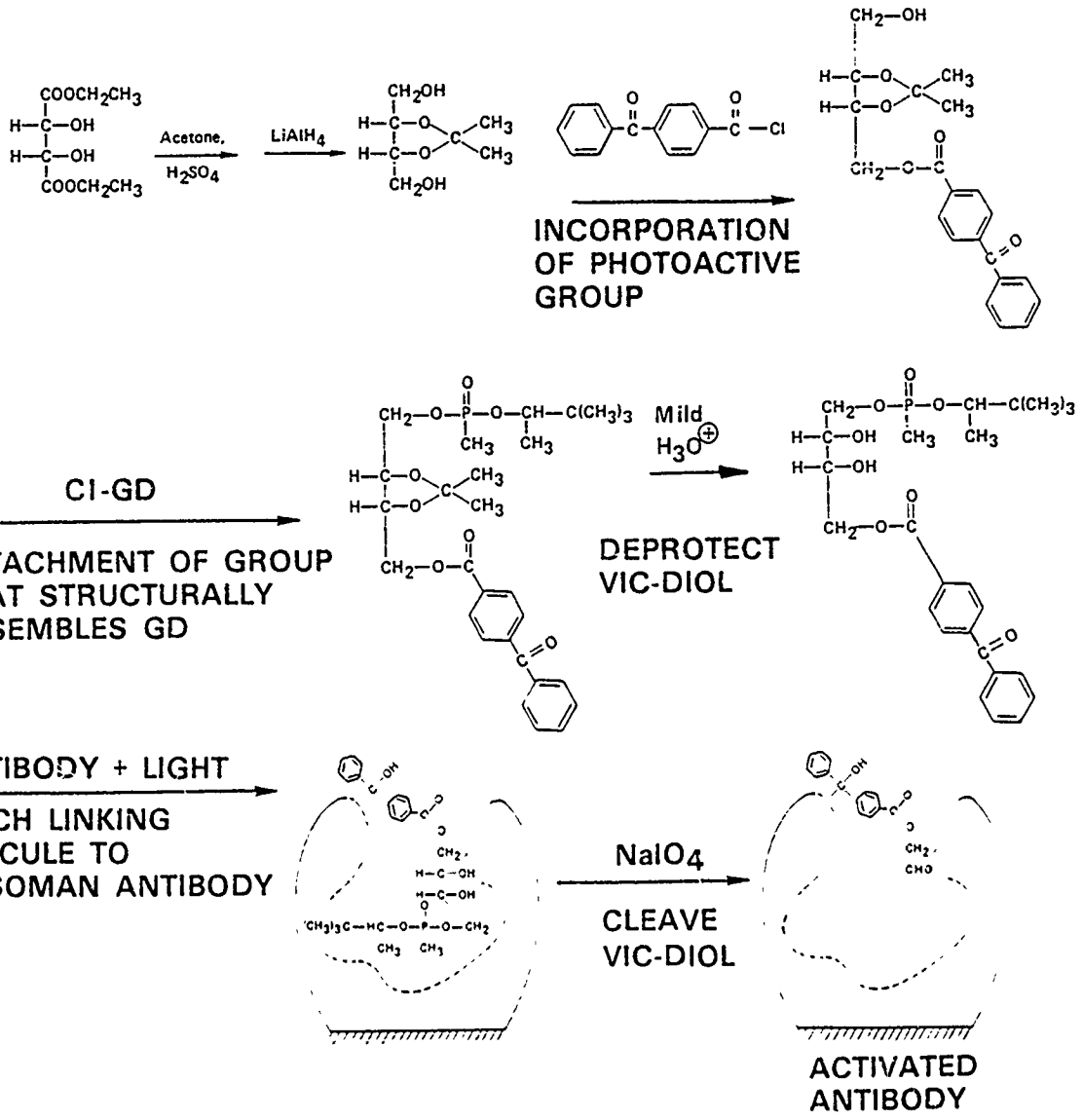
SYNTHESIS OF THE IMMUNOENZYME (BLOCK DIAGRAM)



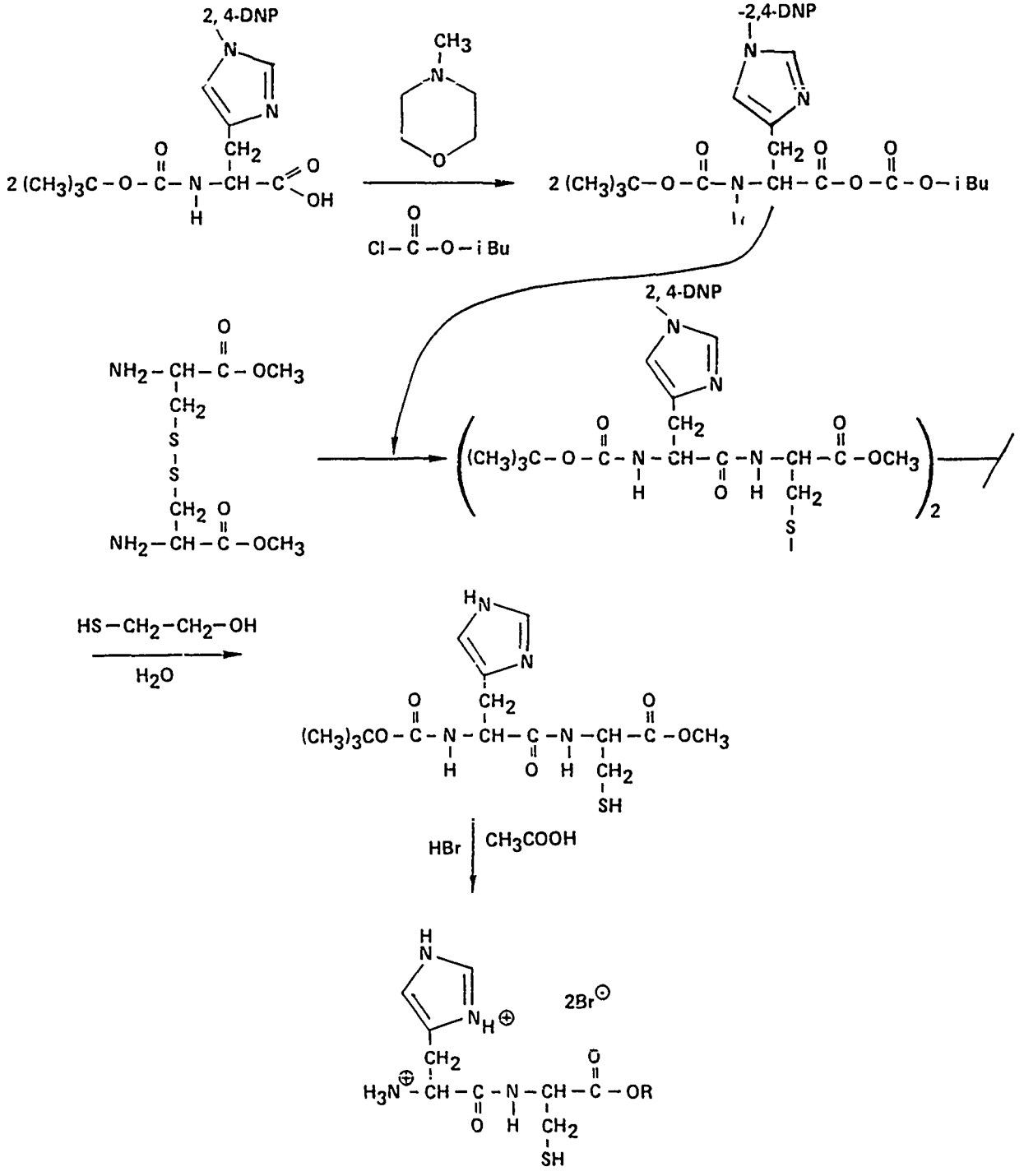
CATALYTIC CYCLE



SYNTHESIS OF ACTIVATED ANTIBODY



SYNTHESIS AT THE CATALYTIC MOIETY



FUTURE OBJECTIVES

1. COMPLETE IMMUNOENZYME SYNTHESIS AND CHARACTERIZATION.
2. DETERMINE IN VITRO CATALYTIC ACTIVITY AGAINST SIMULANTS AND SOMAN.
3. DETERMINE IN VIVO EFFICACY IN LABORATORY ANIMALS.
4. INCREASE CIRCULATING LIFETIME OF IMMUNOENZYME.

ACKNOWLEDGMENTS

This work was supported by the US Army Medical Research and Development Command, Acquisition Division, Fort Detrick, Frederick, Maryland, under Contract DAMD17-82-C-2082. Dr. L. Eric Crane contributed to the design of the synthetic routes. Mr. Roosevelt Rush and Ms. Debra L. Gable performed the laboratory work involved in this contract. Dr. G.M. Anantharamaiah, University of Alabama at Birmingham, assisted in the synthesis of the catalytic moiety. Dr. Kenneth Hunter, Uniformed Services University of Health Science (Bethesda, MD), generously provided the antisoman monoclonal antibody.

DESIGN AND SYNTHESIS OF AN "ENZYME FRAGMENT" FOR WOUND PATIENT DECONTAMINATION

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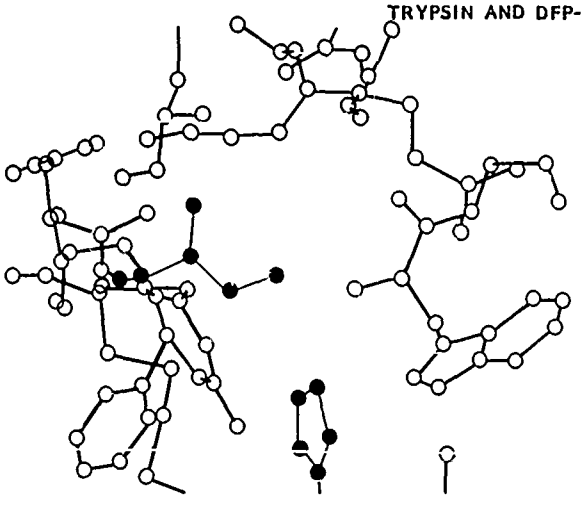
A series of enzymes known to contain "active serines" were tested to determine their rates of reaction (second order rate constants, k_2) with diisopropylfluorophosphate (DFP). Rates were measured against native and denatured ("perturbed") enzymes. The comparison between the native rate (k_{2n}) and the denatured rate (k_{2d}) was to ultimately provide a determination of the relative contributions of the primary and tertiary enzyme structures in the reaction with DFP.

THE RATES OF REACTION OF DFP
WITH SELECTED "ACTIVE SERINE" ENZYMES

ENZYME	NATIVE (k_{2n})	DENATURED (k_{2d})	k_{2d}/k_{2n} (DFP)
Butyrylcholinesterase (Horse Serum)	2.9×10^5	1.0×10^5	35
Carboxylesterase (Porcine Liver)	3.0×10^4	6.4×10^3	20
Elastase (Porcine Pancreas)	3.5×10^3	2.2×10^3	65
Chymotrypsin (Bovine Pancreas)	2.7×10^3	1.9×10^3	70
Acetylcholinesterase (Electric eel)	1.3×10^3	6.0×10^2	45
Subtilisin (*B. amyloliquefaciens*)	6.4×10^2	4.0×10^2	65
Trypsin (Bovine Pancreas)	2.6×10^2	9.7×10^1	35
Thrombin (Bovine Plasma)	3.2×10^1	2.0×10^1	65
Alkaline Phosphatase (*E. coli*)	0	0	0
Phosphoglucomutase (Rabbit Muscle)	0	0	0
Phosphorylase *a* (Rabbit Muscle)	0	0	0

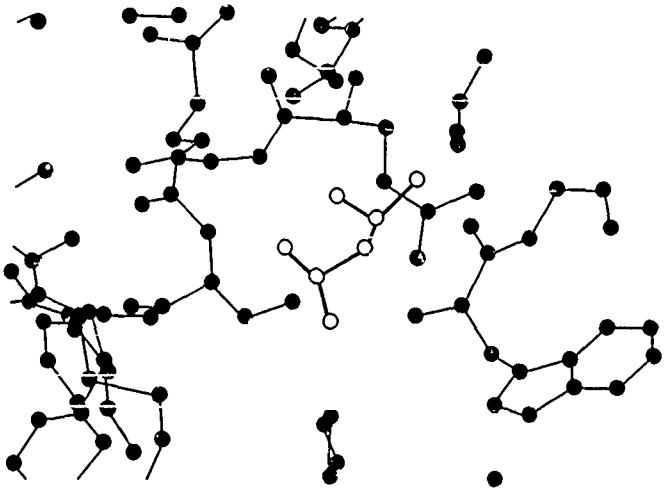
The enzymes were grouped into classes on the basis of their relative reactivities with DFP. Computer-generated models were utilized to determine the necessary determinants in amino acid structure and sequence for the reaction between the "active serine" enzyme and DFP.

TRYPSIN AND DFP-INACTIVATED TRYPSIN



The Trypsin Binding Site

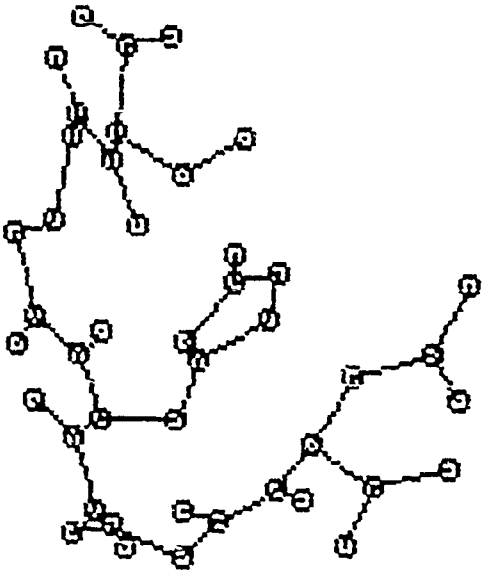
DFP-Inactivated Trypsin



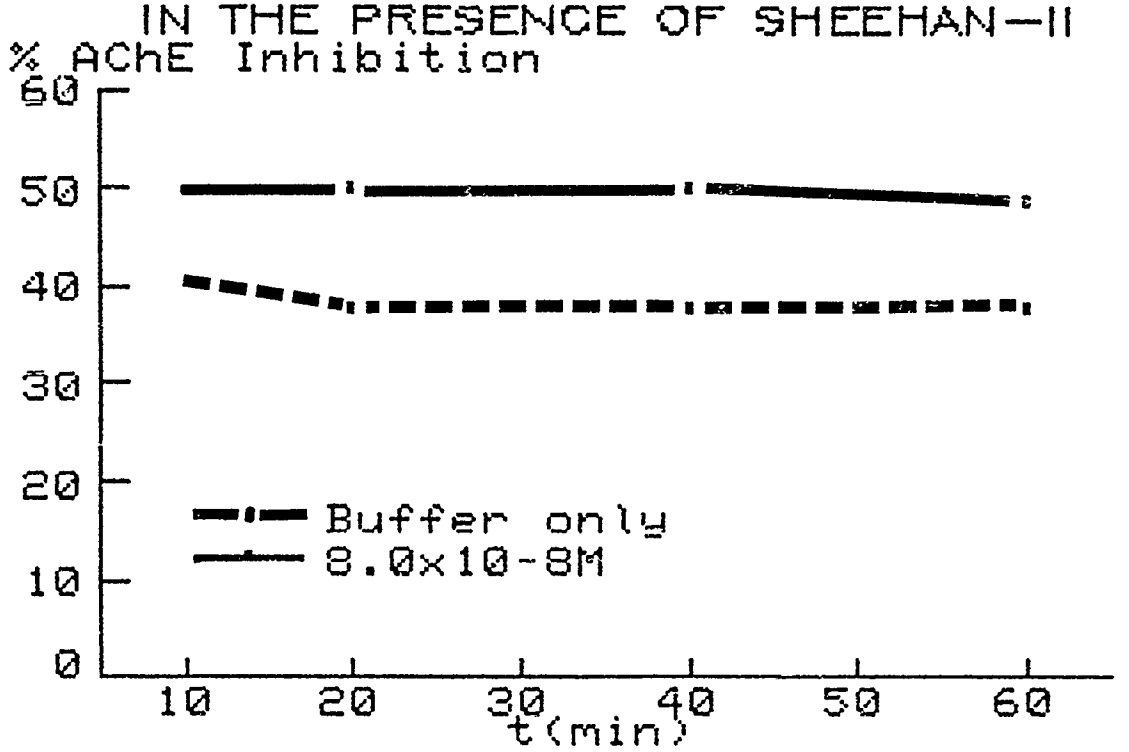
PEPTIDE	SEQUENCE
Sheehan I	THE-ALA-SER-HIS-ASP
Sheehan II	SER-GABA-HIS-GABA-ASP
Kapoor	HIS-ALA-ASP-GLY-SER-PHE
Kitchell I	PHE-GLY-GLU-SER-ALA
Kitchell II	HIS-PHE-GLY-GLU-SER-ALA
Kitchell III	HIS-GABA-SER
Kitchell IV	SER-GABA-ASP
Kitchell V	HIS-GABA-ASP
Kitchell VI	SER-GABA-LEU-HIS-GABA-ASP
Kitchell VII	LEU-SER-GABA-HIS-GABA-ASP
Kitchell VIII	TYR-GABA-HIS-GABA-ASP

THE SHEEHAN II PEPTIDE

(Ser-GABA-His-GABA-Asp)

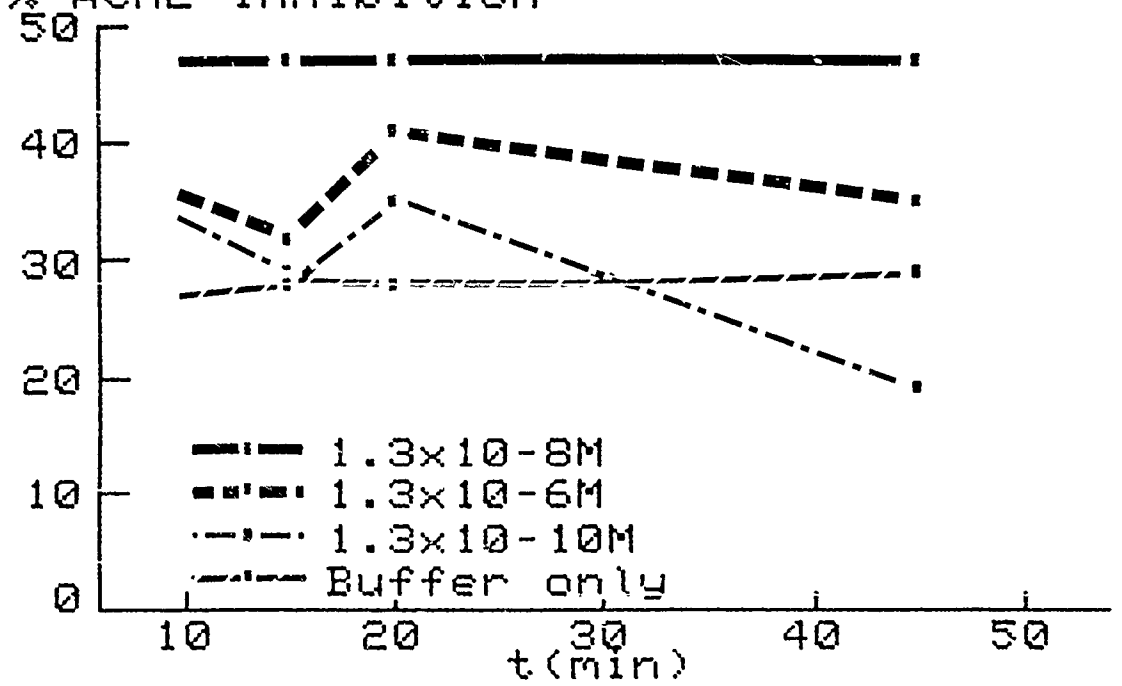


INHIBITION OF ACHE BY DFP
IN THE PRESENCE OF SHEEHAN—II



A DFP solution (4.0×10^{-6}M) was incubated with a solution of the Sheehan II peptide in phosphate buffer, pH 7.0. Aliquot samples of the reaction mixture are removed at various times, t, and assayed for residual AChE inhibition.

INHIBITION OF ACHE BY SOMAN
IN THE PRESENCE OF SHEEHAN—II



A Soman solution (5.0×10^{-9}M) was incubated with a solution of the Sheehan II peptide in phosphate buffer, pH 7.0. Aliquot samples of the reaction mixture are removed at various times, t, and assayed for residual AChE inhibition.

The rate data for reaction of Sheehan II with DFP and Soman indicated that, for both cases, reaction between peptide and organophosphate was not stoichiometric. The Sheehan II peptide reacted with (and thus deactivated) agent in molar concentrations greater than that of the peptide itself. Reaction was rate-limited, however, suggesting that some product inhibition may be responsible for lack of total agent deactivation. ^{31}P and ^{1}H NMR studies were consequently undertaken to deduce the mechanism of peptide:agent reaction.

^{31}P NMR: pH DEPENDENCE ON CHEMICAL SHIFT

$$\text{DFP} \xrightarrow{OH^-} \text{DIP} + F^-$$

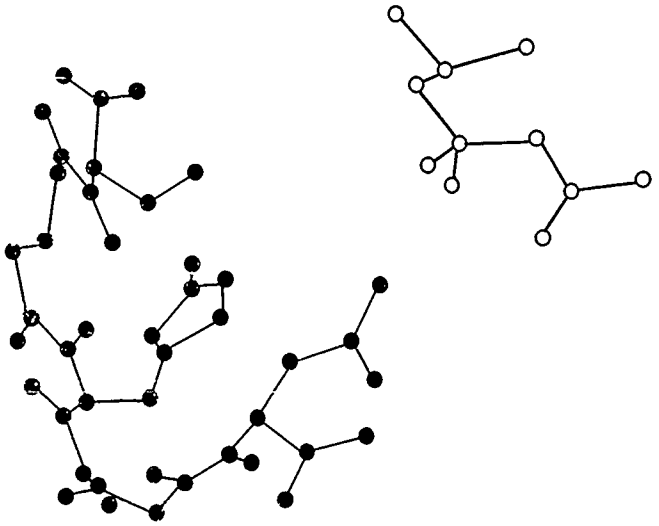
-0.85 ppm (pH 7.9)

$$\text{PEPTIDE} + \text{DFP} \longrightarrow \text{PEP-DIP} + F^- \xrightarrow{OH^-} \text{PEPTIDE} + \text{DIP} + F^-$$

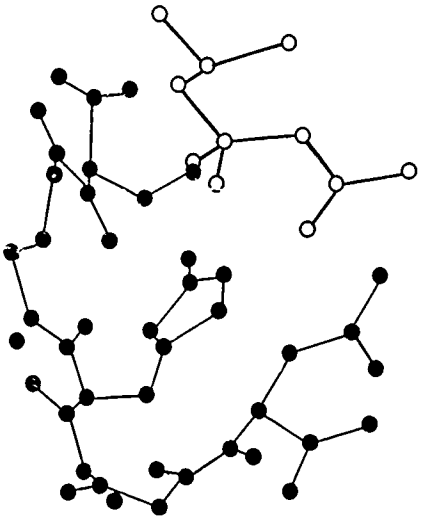
1.15 ppm (pH 2.6) -0.85 ppm (pH 7.9)

The ^{31}P NMR data suggested that base hydrolysis was responsible for the observed peptide "turnover" of DFP, while the ^{1}H NMR spectra strongly suggested the involvement of one of the peptide amino acids (as yet undetermined, although suspected to be serine). A postulated mechanism for the peptide:DFP reaction involves the initial formation of a Sheehan II:DFP complex (with concomitant release of fluoride) followed by hydroxide cleavage to yield free Sheehan II and hydrolyzed DFP ("DIP").

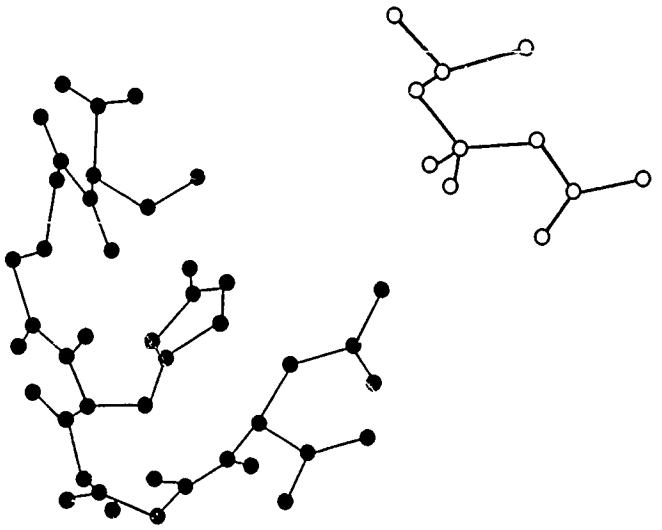
BASE-CATALYZED PEPTIDE HYDROLYSIS OF DFP



Sheehan II + DFP



Sheehan II:DFP



Sheehan II + DIP

SUMMARY:

The need exists for the development of a rapid and safe material for human decontamination applications in cases of agent exposure. Existing chemical agent decontamination procedures are effective in some situations, but are considerably irritative and toxic for human decontamination. The preparation of a formulation which is specific for agent and suitable for topical use would find many potential military applications.

The goal of this laboratory program is to produce a material for wound patient decontamination. This is to be accomplished via the development of a product which mimics the binding site of the cholinesterase enzymes, the biological target of these agents. A peptide ("enzyme fragment") can be designed with spacing and directionality coincident with the protein binding site such that selective agent recognition is retained. Use of an enzyme fragment would hold the advantage over use of the enzyme itself by virtue of the projected formulation stability and immunocompatibility.

The development objective is to be achieved via investigation in three interrelated program areas. The first deals with the study of the relative reactivities of the cholinesterase and related enzymes with a model organophosphate (DFP). The second area addresses the correlation of these results with enzyme structural studies to lead to the design, synthesis, and testing of appropriate peptides for organophosphate decontamination. The third program area relates directly to the formulation of a wound patient decontamination reagent which is based upon the incorporation of the active peptide into a suitable pharmaceutical preparation.

At this point in the development program, work has proceeded in the first two areas, i.e., the enzyme and peptide studies. The results of the structure/activity studies for reaction of candidate enzymes with DFP are presented here. This correlative work is then embellished to illustrate the impact on peptide design, synthesis, and testing. The overall progress in the elucidation of decontamination mechanisms as they relate to this development program is summarized.

This work supported in part by the U.S. Army Medical Research and Development Command under Contract No. DAMD-17-82-C-2244.

FUTURE DIRECTION:

Present efforts are focused upon additional elucidation of the mechanism of Sheehan II:DFP reaction. The primary impact of this work will be in the areas of further peptide design, synthesis, and testing. Ultimately, that peptide which proves to be the most promising will be incorporated into an appropriate formulation for wound patient decontamination.

PROGRESS IN ISOLATING A CELL LINE SECRETING HIGH LEVELS OF HUMAN ACETYLCHOLINESTERASE

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BIO-RESPONSE, INC., 1978 West Winton Avenue, Hayward, CA 94545

Abstract

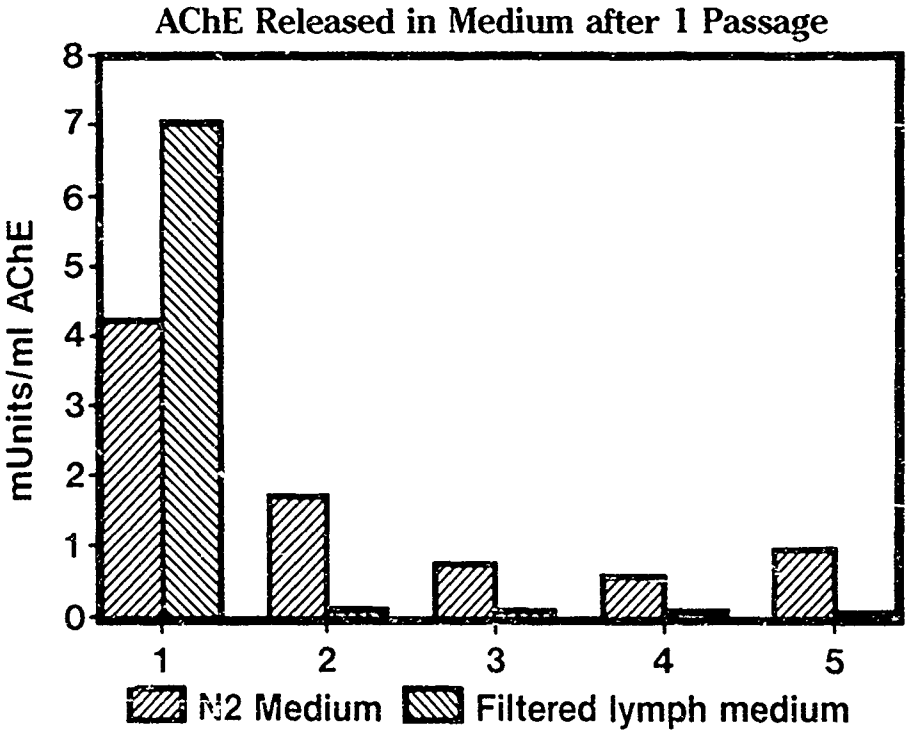
Acetylcholinesterase (AChE) is one of many potentially valuable enzymes and proteins secreted at very low levels by human cell lines but which might be produced in significant quantities *in vitro* using a large scale mammalian cell culture system.

Our aim is to first isolate a high producing variant and then grow the variant in our Mass Culture System. Using a new radiometric immuno-adsorbant solid-phase assay (RISA), we screened various human cell lines of neuronal, glial, lymphocytic and muscular origins. Candidate cells were adapted to growth in defined media or media supplemented with a low molecular weight filtrate of fresh bovine lymph free of any contaminating esterase activities. AChE secretion rates for these cells in normal static culture were found to range from 1 to 10 milliunits of true AChE activity per 10^6 cells per day (approximately 20-200 picograms of active AChE monomer per ml).

In an effort to increase the yield of AChE per cell, we have proceeded with two versions of our Cell Isolation Technique. Our premise has been that we could select the few cells of a sub-population which, by spontaneous gene amplification mechanisms, for example, have become higher producers of AChE than cells in the starting population. In the first approach, we have begun encapsulating cells within agarose beads (at roughly one cell per bead) together with sheep red blood cells derivitized with either a specific ligand (an AChE inhibitor) or a monoclonal antibody (AE-1) to capture the secreted enzyme. As a result, those beads containing the most productive cells should accumulate more AChE on the surfaces of the trapped red blood cells. By introducing specific developing antibodies followed by complement, hemolysis can be induced in those beads containing threshold levels of antigen (AChE)-antibody complexes on their red blood cells. To date, these methods have been applied to model systems in which we have added low levels of AChE to red blood cells in beads. Beads with lysed erythrocytes can then be readily separated from the original population using a density gradient centrifugation technique. In model systems, one 'positive' bead can be isolated from every 10,000 in the original mixture. Viable cells can be rescued from the isolated beads, grown up and re-selected to enrich for subpopulations maximally secreting AChE.

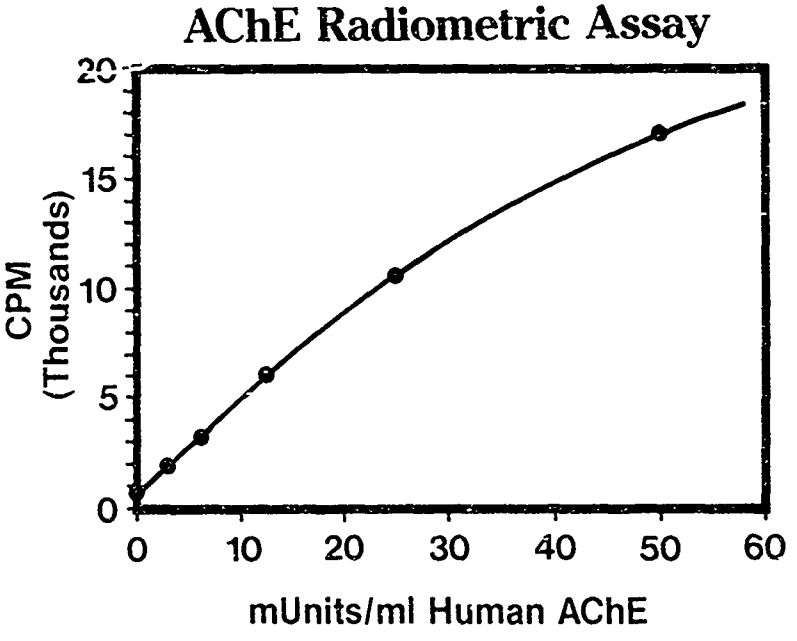
In the second approach, we are developing the capability to use a fluorescence activated cell sorter (FACS) to sort individual beads containing single cells on the basis of the quantity of AChE that has been secreted within each bead. This separation is made possible by immobilizing specific ligands or monoclonal antibodies within the beads such that they capture and accumulate AChE secreted by the single cell in each bead. By introducing a fluorescently-labeled ligand, or alternatively, an antibody, both of which bind to captured AChE, we expect to develop fluorescence intensity in each bead which is proportional to the amount of secreted AChE. In model studies, fluorescent beads can be readily sorted, which would permit positive cells to be isolated, grown up and re-sorted to enrich for high secretors. In addition to our efforts to select for high secretors among constitutive producers, we also intend to report on our attempts to use these techniques to select DNA-transfected cells which express (and secrete) high levels of human AChE.

HUMAN AChE SECRETED BY VARIOUS CELL LINES



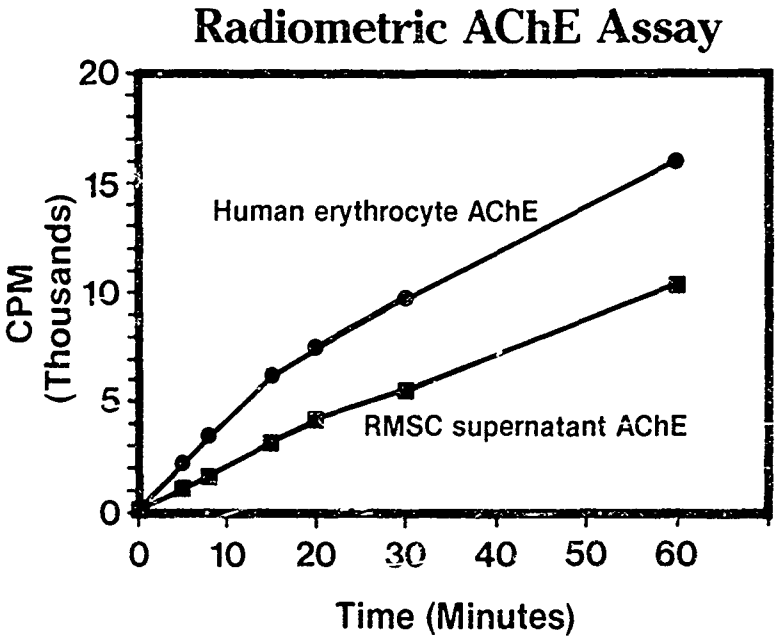
Among the various cell lines screened for the secretion of human AChE, the A-204 rhabdomyosarcoma muscle cell line proved to be the most consistent secretor of enzyme. To avoid high background levels of esterase activities in serum, cells were adapted to growth for limited periods of time in N2 defined medium or medium supplemented with a low molecular weight filtrate of fresh bovine lymph. Cell culture supernatants were then screened at confluence for AChE production using the radiometric assay described below. Human cell lines tested were: 1) A-204 rhabdomyosarcoma; 2) SK-N-MC neuroblastoma; 3) SK-N-SH neuroblastoma; 4) U-87 MG astrocytoma; 5) U-138 MG glioblastoma.

Figure 2a.



Radiometric AChE assay modified from Rotundo and Fambrough (1979) to increase sensitivity for detecting AChE in cell culture supernatants. Standard curve using crude human erythrocyte AChE. ^{14}C-ACh at mCi/mmole; final [ACh] = 0.1mM in 100μl of 0.15M NaCl, o.1M sodium phosphate pH7.0, 0.25mM EDTA, 1% Triton X-100, and 0.02% sodium azide at 37°C.

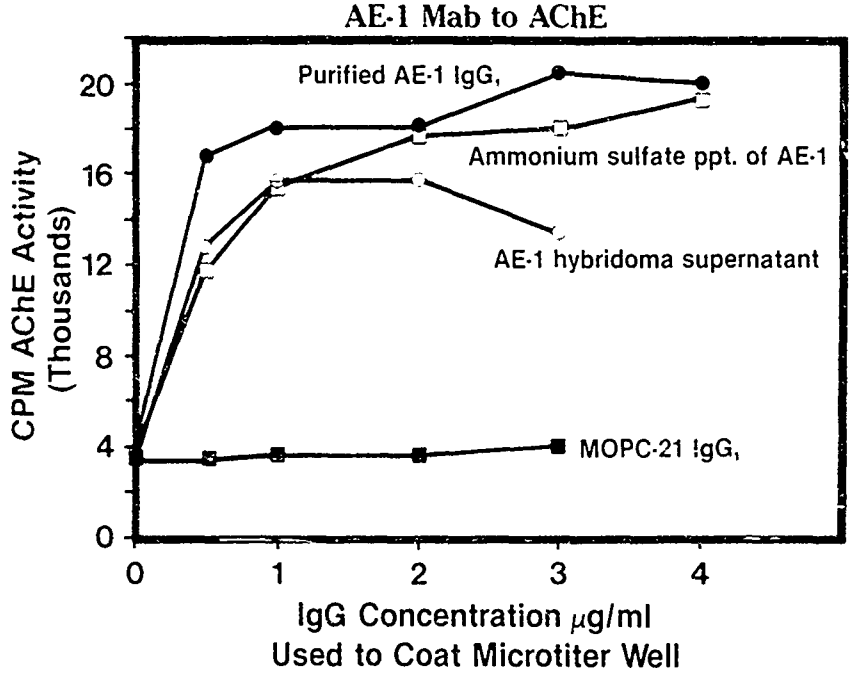
Figure 2b.



Modified radiometric assay described above is nearly linear with respect to time. Human erythrocyte AChE (10 mUnits/ml) vs. test sample of RMSC cell culture supernatant.

Figure 2c.

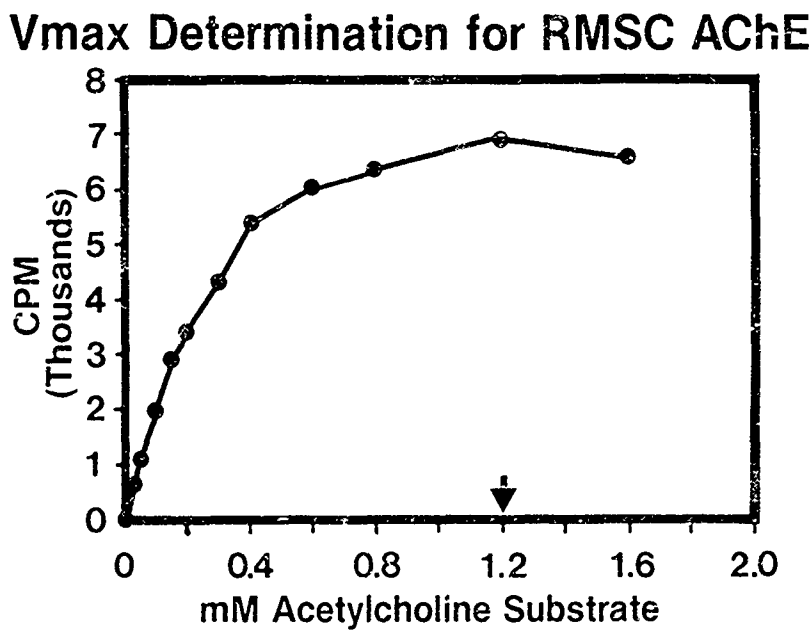
Radiometric Immunoadsorbant Solidphase Assay (RISA)



Various preparations of monoclonal antibody (AE-1) adsorbed to PVC microtiter plates will bind human AChE. AE-1 IgG_1 fractions were used to coat microtiter wells at concentrations indicated. After washing and blocking any remaining sites with 1% BSA-PBS, the plates were incubated with crude human erythrocyte AChE (10 mUnits/ml) for 1 hr at 23° C. The plates were washed again and incubated for 20 hr at 37° C with ^{14}C-ACh substrate under conditions described for our modified radiometric assay. Hydrolysis rates for the bound AChE were determined from the levels of ^{14}C-acetate released into the reaction medium.

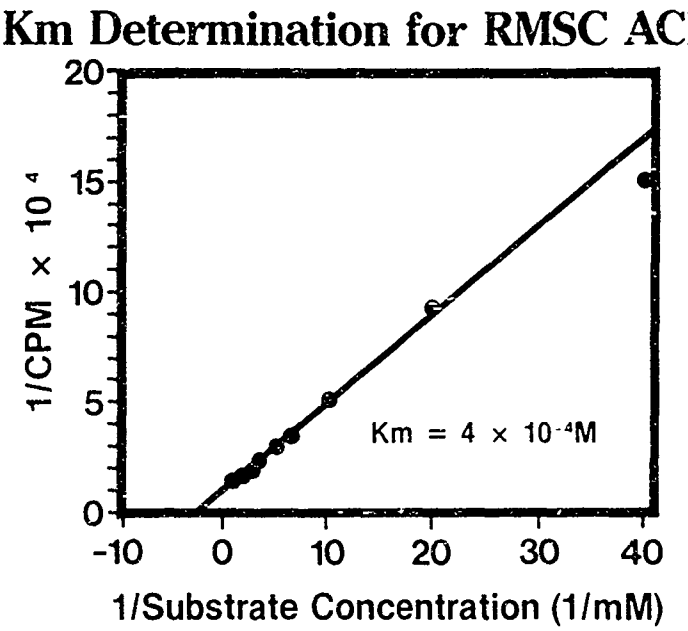
CELL LINES SECRETE TRUE AChE

Figure 3a.



The concentration of ACh substrate required to give maximal rates of hydrolysis (Vmax) for secreted AChE was found to be 1.2 mM using the radiometric assay. The AChE activity from RMSC cells began to exhibit the characteristic substrate inhibition at concentrations of ACh above 1.2 mM.

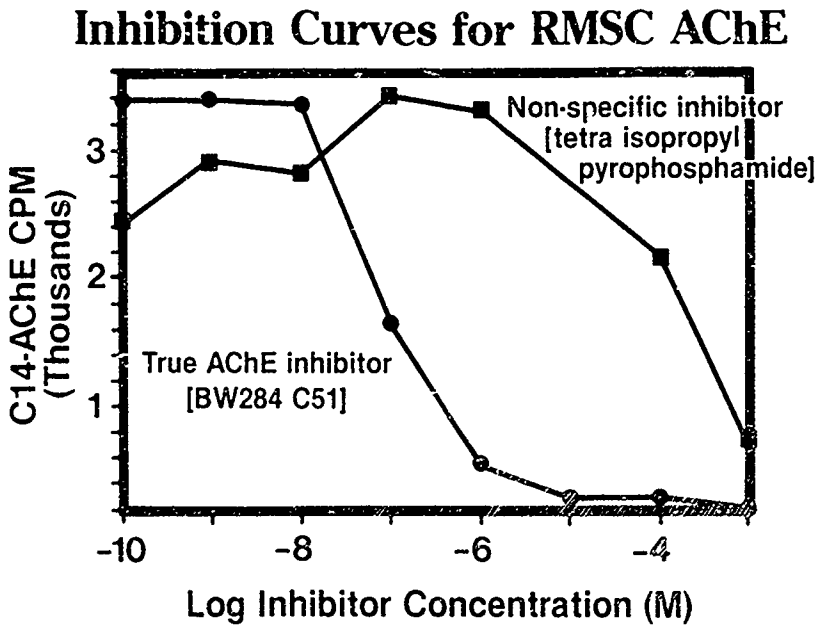
Figure 3b.



The Michaelis constant, Km, for the AChE secreted by RMSC cells was calculated to be 0.4 mM by analysis of this Lineweaver-Burk plot of a radiometric assay.

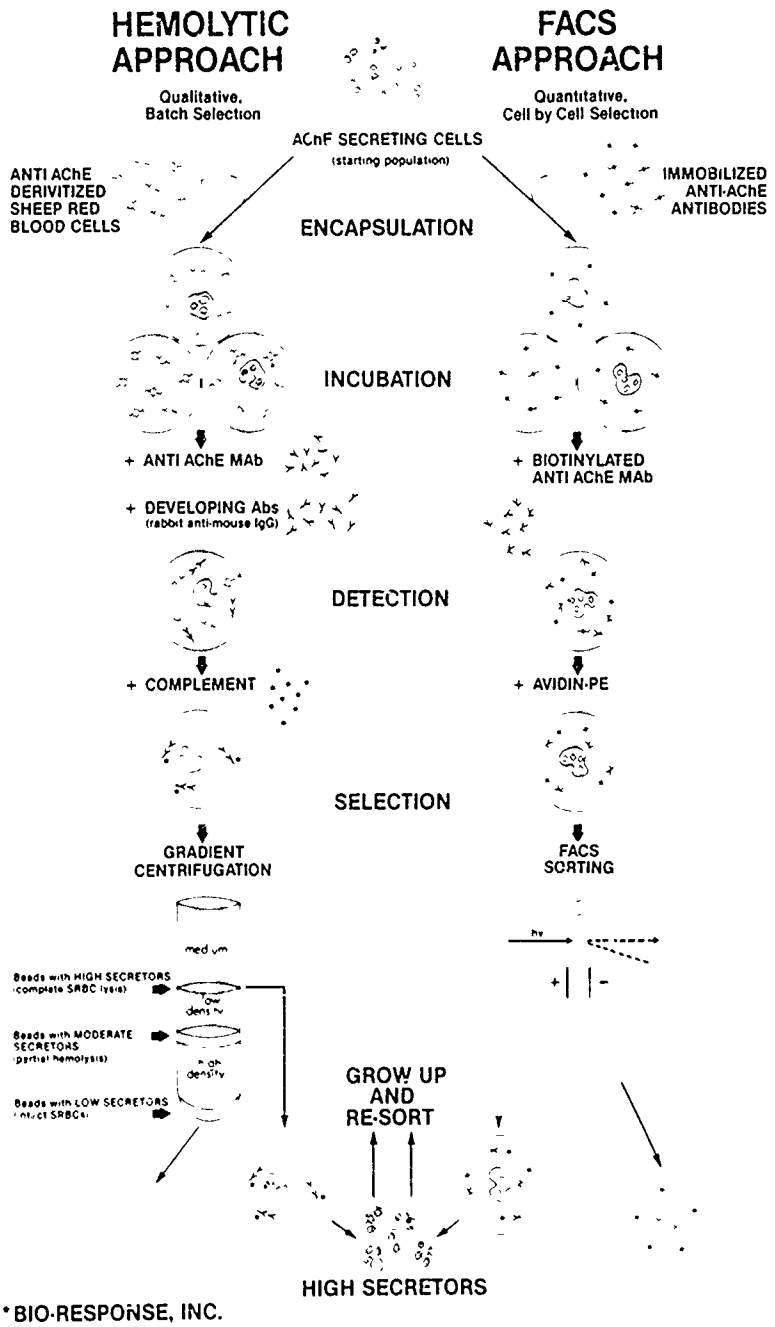
CELL LINES SECRETE TRUE AChE

Figure 3c.



Rhabdomyosarcoma supernatant AChE was tested for inhibition using the radiometric assay with the indicated concentrations of BW 284 C51, a true AChE inhibitor, and Iso-OMPA, a non-specific esterase inhibitor. The concentration required for 50% inhibition with BW 284 C51 was 10^{-7} molar, whereas for the non-specific inhibitor, the apparent Ki was around 10^{-4} molar.

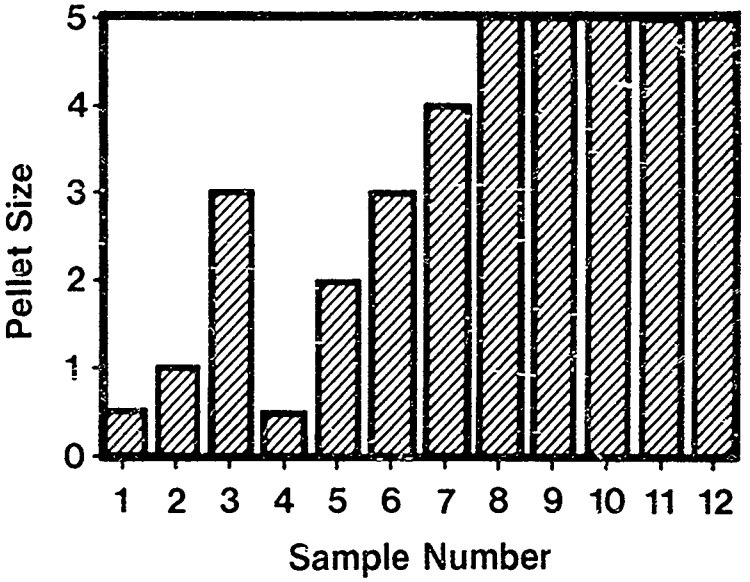
CELL ISOLATION TECHNIQUES



*BIO-RESPONSE, INC.

HEMOLYTIC C.I.T. DATA

SRBC—Ligand Hemolysis by AChE



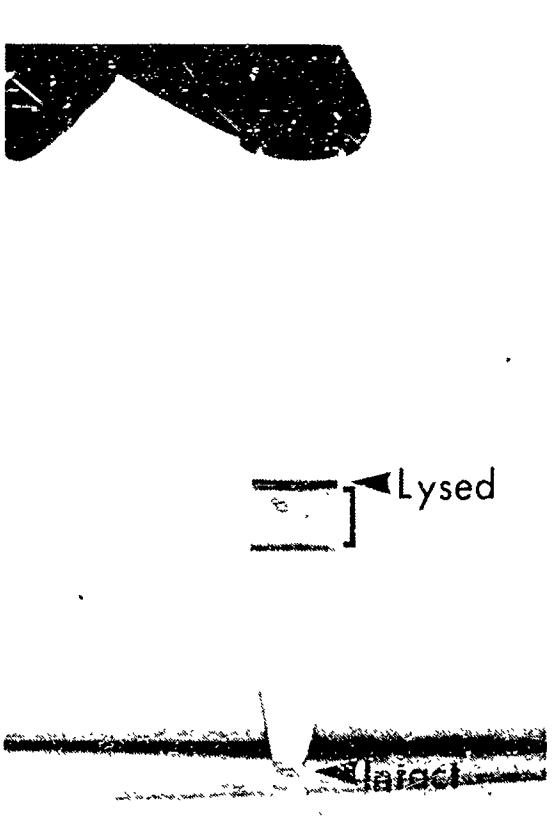
In one model study, sheep red blood cells were derivitized with an AChE inhibitor, p-nitrophenyl methylphosphonochloridate, which binds covalently to the esteratic site on the enzyme. The ligand-derivitized SRBC's were encapsulated inside beads and incubated with AChE secreted by RMSC cells. The beads were then incubated with AE-1 monoclonal antibody followed by secondary rabbit anti-mouse IgG. Exposure to adsorbed guinea pig complement induced hemolysis of SRBCs which had captured sufficient AChE. The beads with many lysed SRBC can be harvested by a density gradient centrifugation technique (Fig 5c). 1-6, varying concentrations of AChE, AE-1 and R anti-M IgG; 7-12, minus AChE controls.

Figure 5b.

SRBC Trapped in Beads

HEMOLYTIC C.I.T. DATA

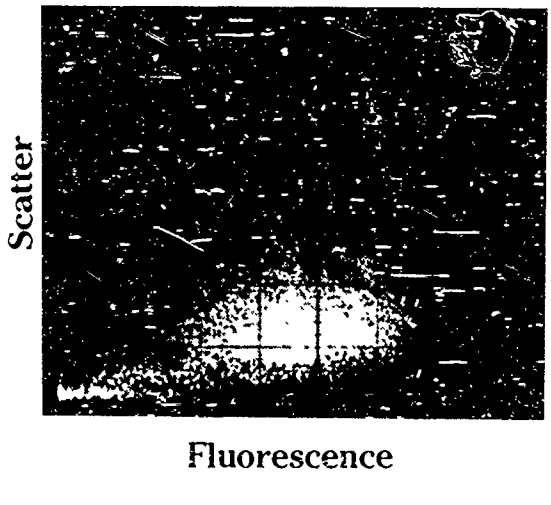
Figure 5c.



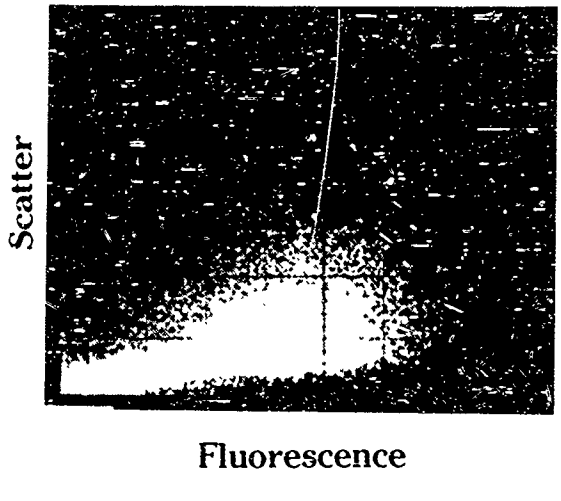
A preliminary experiment in which agarose beads containing completely lysed SRBC's (top band, colored with free hemoglobin) in beads could be separated from beads containing partially lysed populations of SRBC's (intermediate band). The pellet would have contained all the beads remaining with intact SRBC's (negative population of cells).

FACS APPROACH — MODEL STUDIES

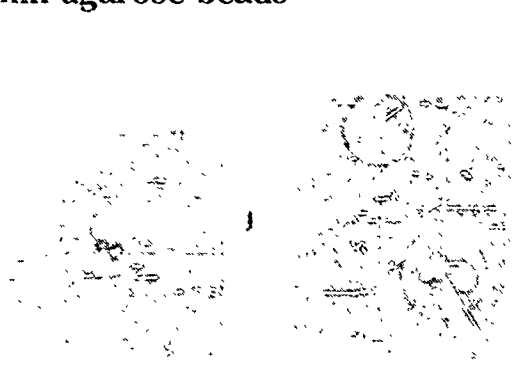
Fluorescentiy Labelled Cells Inside Beads can be Sorted



Fluorescent/Scatter histogram for human T cells labelled with FITC-anti-human transferrin receptor MAb (FITC-α-TR)

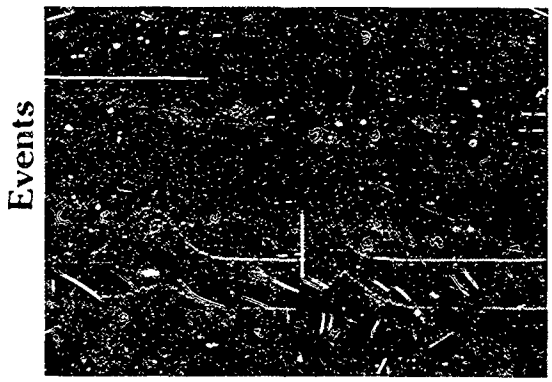


Fluorescence/Scatter histogram for same cells labelled with FITC-α-TR after encapsulation within agarose beads



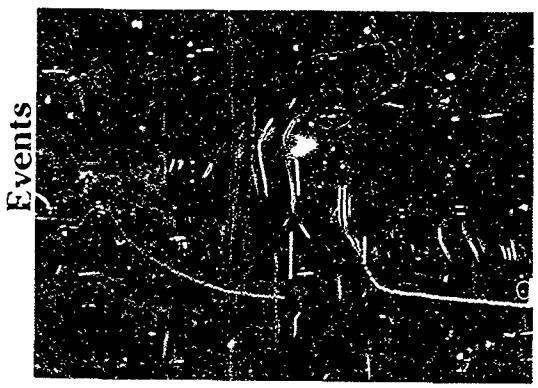
FACS APPROACH — MODEL STUDIES

Fluorescently Labelled Cells Inside Beads can be Sorted



Left: Forward scatter profile for cells alone (upper trace) Forward scatter profile for encapsulated cells (lower trace)

Right: Fluorescence profile for cells alone (upper trace) Fluorescence profile for encapsulated cells (lower trace)



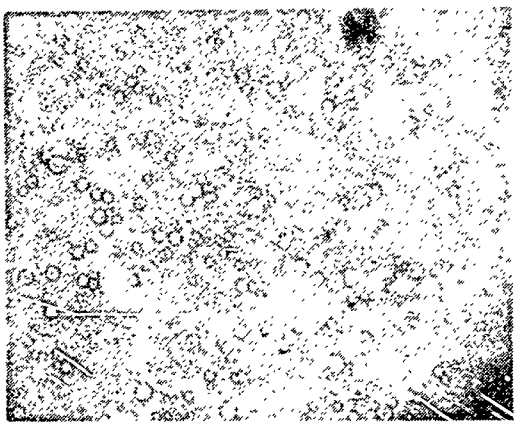
Left: Sort window for scatter from encapsulated cells

Right: Sort window for fluorescence from encapsulated cells

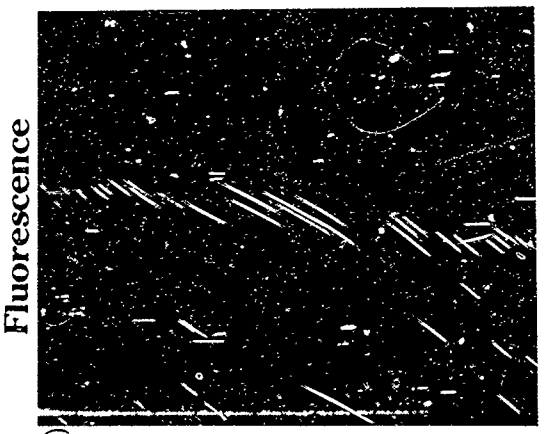
Results: 1×10^7 cells in beads sorted (60% viable) from 3×10^7 cells in beads (starting population, 95% viable)

FACS APPROACH — MODEL STUDIES

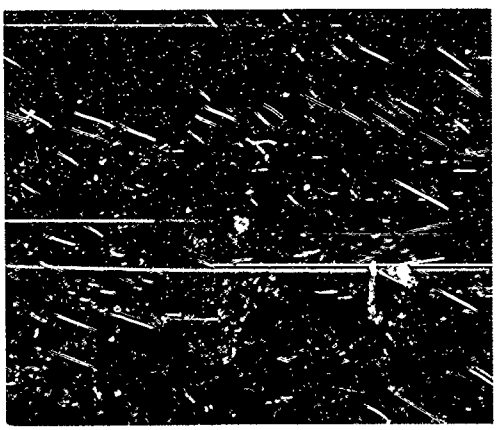
SORTING OF AGAROSE BEADS CONTAINING TRAPPED FLUORESCENT LATEX PARTICLES



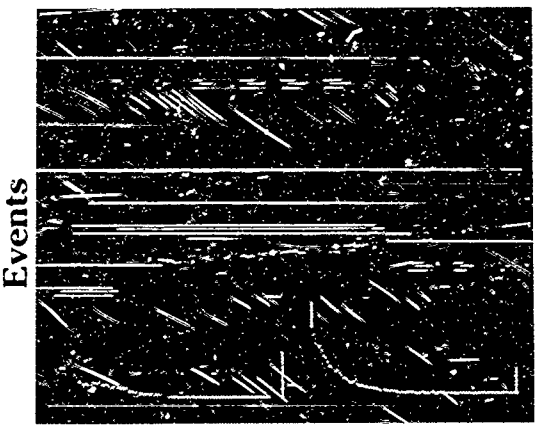
Ⓐ Light Micrograph



Ⓒ Scatter Dot Plot

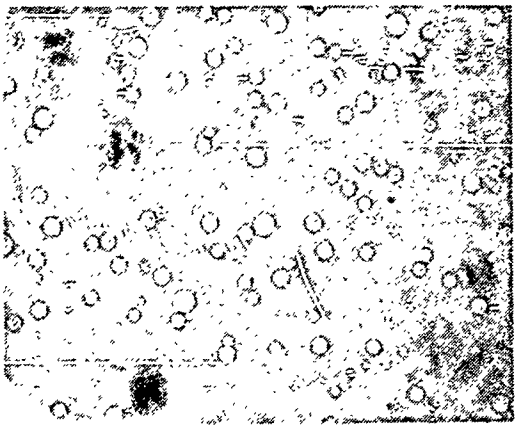


Ⓑ Fluorescence Micrograph

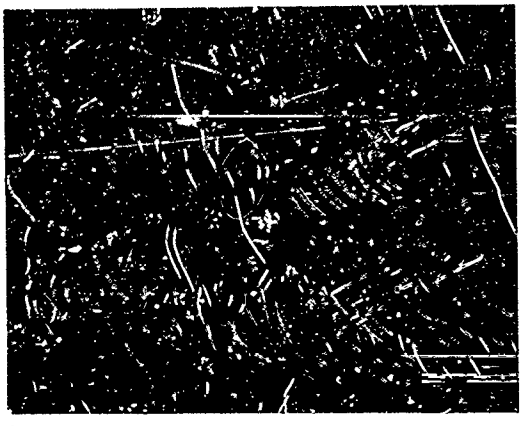


Ⓓ Scatter Fluorescence Histograms

FACS APPROACH — MODEL STUDIES



Ⓔ Light Micrograph



Ⓕ Fluorescence Micrograph

As a demonstration that fluorescent beads can be sorted from non-fluorescent beads, agarose beads were loaded with fluorescent latex particles (0.1 um) and diluted 1:1500 with beads containing unlabeled latex particles (micrographs A and B). FACS analysis confirmed that the fluorescence was confined to light scattering bead structures (dot plot, C). Beads were sorted by triggering on fluorescence as indicated by the gating window shown in D. Micrographs of the recovered "positive" beads (E and F) revealed that greater than 80% of the beads contained fluorescent particles; this represents a 1200 X enrichment.

Conclusions

1. Methods are being developed for the isolation of subpopulations of cells which secrete higher levels of specific proteins (such as human AChE) than cells in the original starting population.
2. A batch approach based on the encapsulation of secretory cells and sensitized red blood cells within agarose beads may be useful for obtaining populations of cells enriched for high secretors.
3. A more quantitative approach based on encapsulating the enriched populations and using fluorescence signals to sort for high secretors may serve to isolate transformed cells or DNA-transfected cells which secrete high levels of human AChE.

SYNTHESIS OF SOMAN ANALOG/PROTEIN CONJUGATES FOR USE AS IMMUNOGENS

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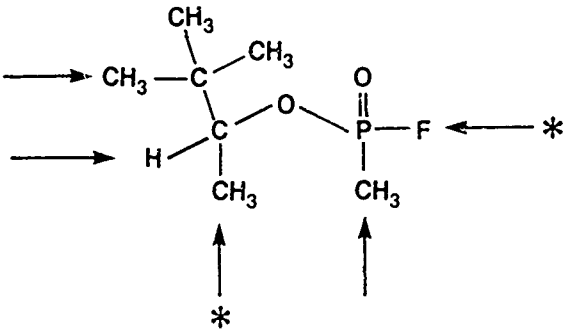
Research Triangle Institute, Research Triangle Park, NC 27709

ABSTRACT

Soman is not immunogenic *per se*, but must be coupled to a large molecule such as a protein in order to stimulate formation of antibodies which bind to soman. Introduction of a covalent bond linking a small molecule to protein results in some change in the structure of the small molecule. To be as immunologically innocuous as possible, the linking group between soman and protein should be relativeiy small in volume, uniform in structure and should facilitate extension of the small molecule component away from the surface of the protein. Since no linking group can provide ideal properties, the position and structure of the link should be varied systematically in order to obtain antibodies with the desired affinity and selectivity. We are currently undertaking such a study in which the linking groups are composed of (a) methylene units, (b) acetylenic units and (c) perfluoromethylene units terminating in a carboxyl group. The position of the link is also being varied.

Acids are conjugated to bovine serum albumin (BSA), bovine thyroglobulin (BTg) and keyhole limpet hemocyanin (KLH) by conversion of the carboxylate to a mixed anhydride (isobutyl-chloroformate/triethylamine/dioxane) followed by reaction with the protein in aqueous solution (aqueous ethylene glycol for KLH). The resulting conjugates are separated from unreacted phosphonate by gel filtration chromatography, ultrafiltration or dialysis. Incorporation of the phosphonate into the protein is determined by phosphorus analysis, carried out in a sequential plasma emission spectrometer. P-31 NMR indicates the integrity of the phosphonate group. Use of P-31 NMR for quantitation of phosphonate incorporation into protein is being investigated in both the presence and absence of a free radical (to decrease and level relaxation times). Molar ratios of hapten:protein range from 4:1 to 26:1 for BSA, from 39:1 to 211:1 for BTg and incorporation into KLH ranges from 0.03 mmole/g of protein to 0.3 mmole/g protein. One to 1.5 g of each conjugate is prepared. (Work supported by Contract No. DAMD-17-84-C-4096.)

Introduction: Position of Hapten-Protein Link

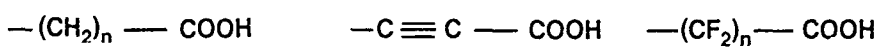


1

Soman is not immunogenic *per se*, but must be coupled to a large molecule such as a protein in order to stimulate formation of antibodies which bind to soman. The arrows in the above structure point to groups which can be substituted with moieties which can link soman to protein. The position of the linking group will influence the selectivity of the resulting antibodies. This position should therefore be systematically varied. The * indicates positions currently being studied.

Because of hazards associated with the presence of a P-F moiety in phosphonate esters and because of the potential reaction with proteins to yield undefined conjugates, P-F was replaced with P-OCH_3.

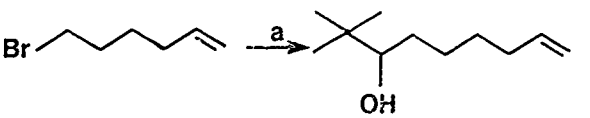
Introduction: Structure of Hapten-Protein Link



2 3 4

To be as immunologically innocuous as possible, the linking group between soman and protein should be relatively small in volume and uniform in structure. It should also facilitate extension of the small molecule component away from the surface of the protein. Linking units currently being investigated include polymethylene, acetylene and polyperfluoromethylene. The linking groups terminate in a carboxyl group which can be activated to react with amino groups on proteins.

Hapten Synthesis: Methylene Link



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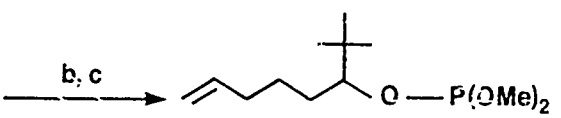

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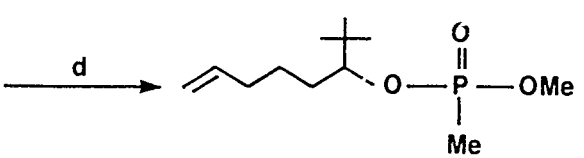

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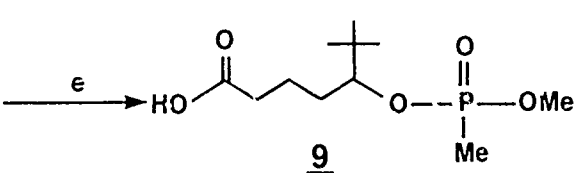
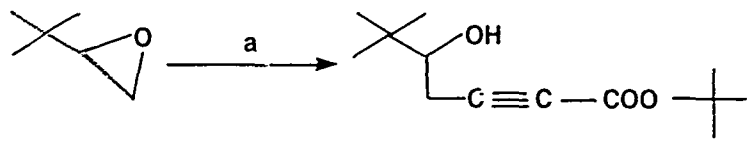


9

(a) Mg; Pivalaldehyde
(b) PCl_3, Et_3N
(c) MeOH, Et_3N
(d) MeI
(e) RuO_2, $NaIO_4$

Hapten Synthesis: Acetylene Link



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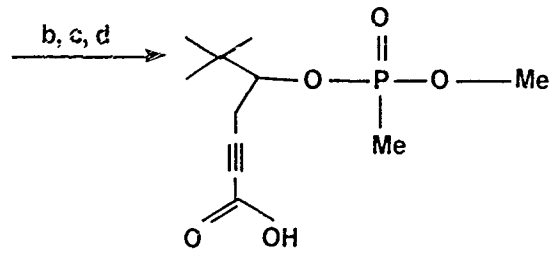


21

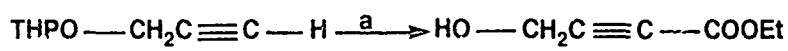
(a) $HC \equiv C-CO_2Bu_t$, n-BuLi, $BF_3 \cdot Et_2O$

(b) $MeP(O)Cl_2$, Et_3N, benzene

(c) MeOH, Et_3N, benzene

(d) Trifluoroacetic acid

Hapten Synthesis: Acetylene Link



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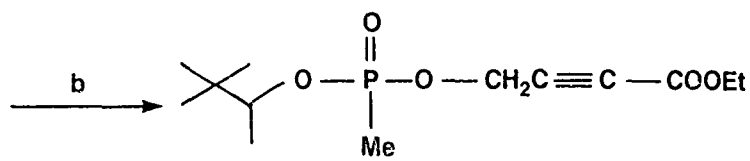

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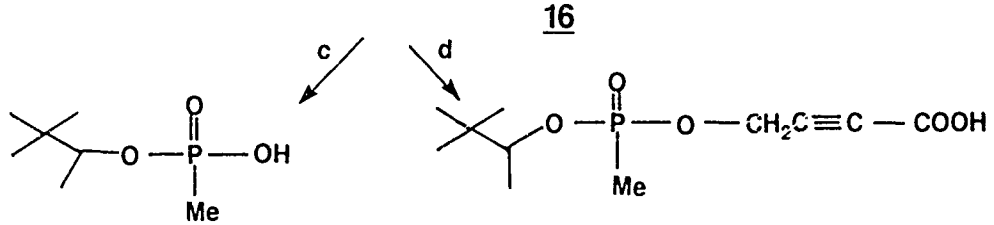


17 18

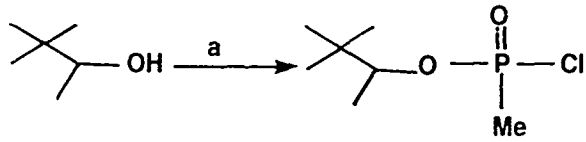
(a) n-BuLi; ClCOOEt; H^+

(b) Pinacolyl methylphosphonyl chloride

(c) Acid or base hydrolysis

(d) Pig liver esterase

Hapten Synthesis: Methylene Link



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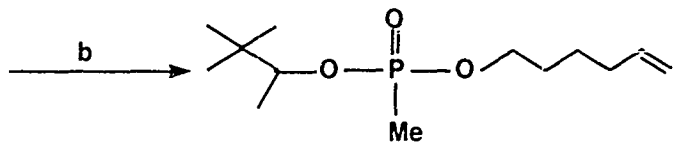

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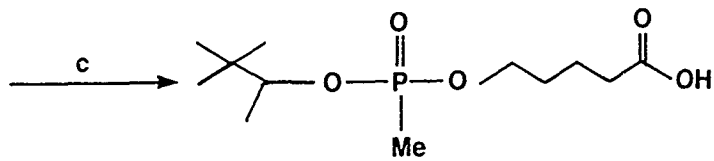
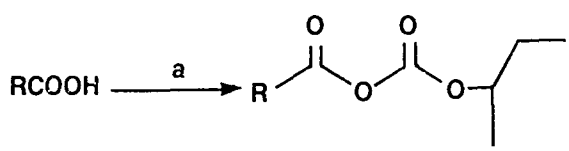


13

(a) $MeP(O)Cl_2$, Et_3N (c) RuO_2, $NaIO_4$

(b) 5-Hexen-1-ol, KH

Conjugation to Protein



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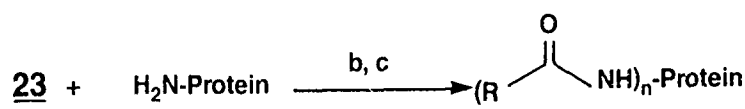


24 25

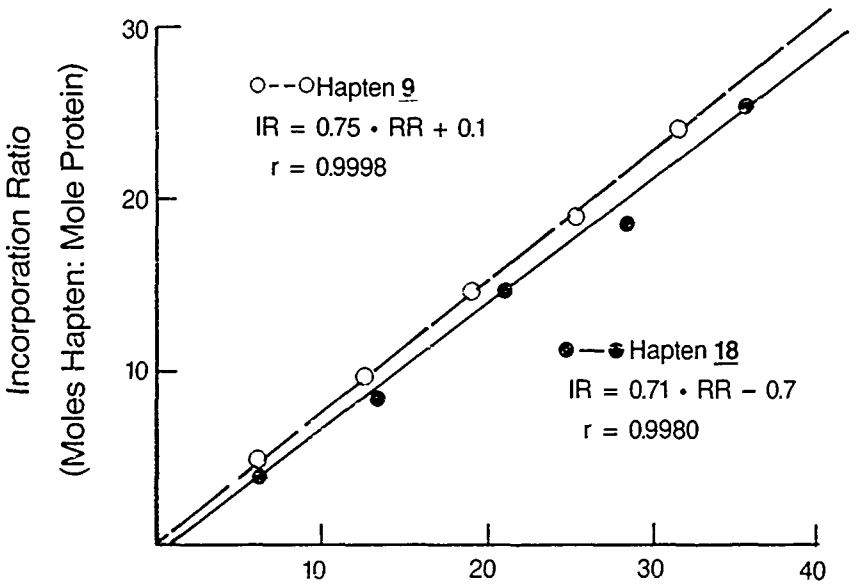
(a) Et_3N, i-butyl chloroformate, dioxane

(b) Aq. NaOH

(c) Purification by dialysis, gel chromatography or ultrafiltration

Proteins are bovine serum albumin (BSA)
bovine thyroglobulin (BTg)
keyhole limpet hemocyanin (KLH)

Incorporation of Phosphonate Ester Into BSA



Incorporation was determined by analysis of phosphorus in the conjugate. P was measured in a sequential plasma emission spectrometer. The average absolute error of measurement (based on analysis of mixtures of pure hapten with BSA) was 4.7%, with errors ranging from −4.5% to +12.4%.

Molar Incorporation Ratios Obtained

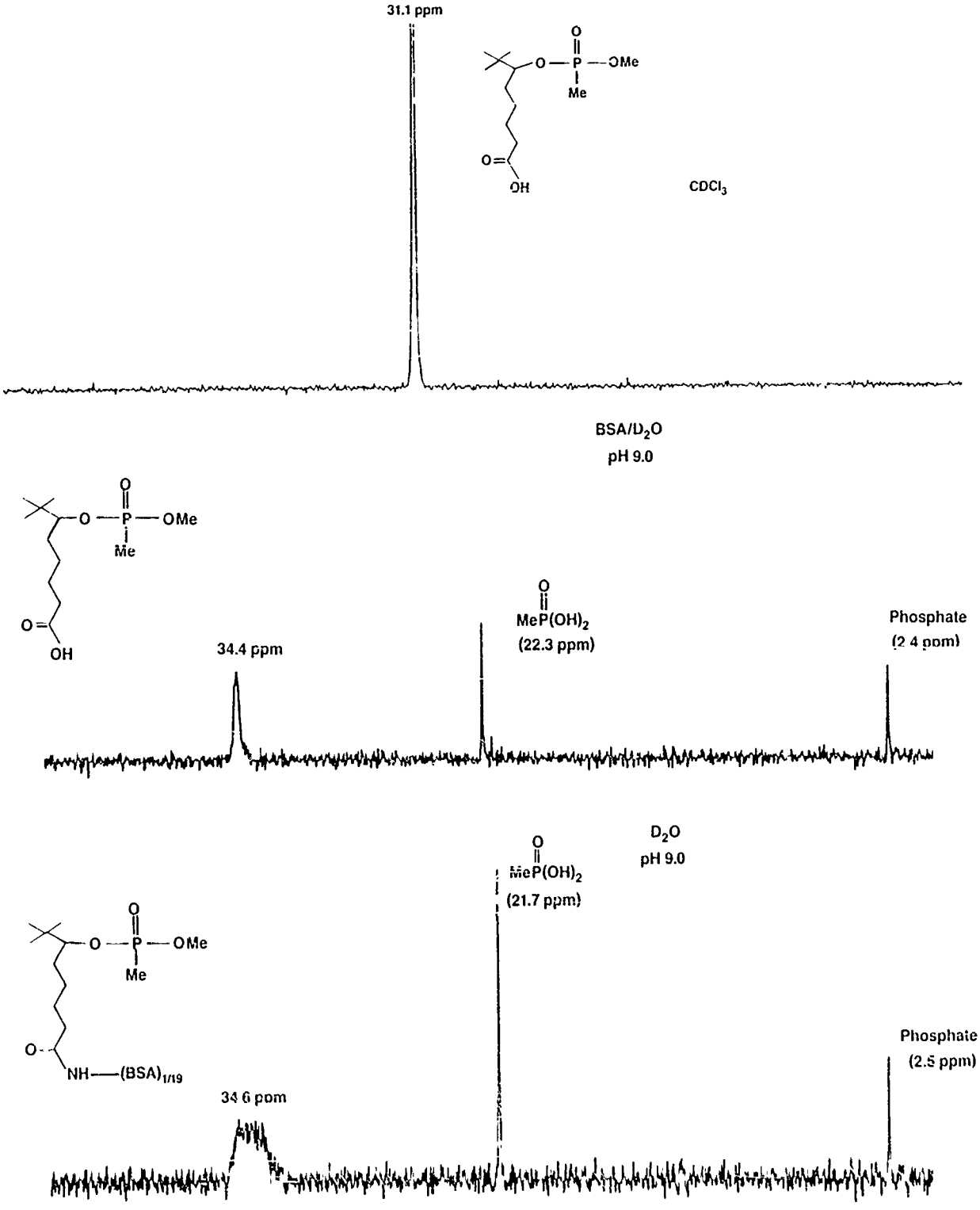
Hapten	Protein		
	BSA	Btg	KLH[a]
9	25.7	146	1635
	19.4	136	1300
	14.7	108	1080
	8.4	79	509
	4.2	40	146
18	24.0	211	—[b]
	19.0	123	
	14.3	107	
	9.4	79	
	4.9	39	

[a]Based on average MW of 5.25×10^6 daltons
[b]In progress

CONCLUSIONS

1. Syntheses of soman analogs with a substituent terminating in a carboxylic acid function have been carried out.
2. These analogs can be conjugated to proteins (bovine serum albumin, bovine thyroglobulin, keyhole limpet hemocyanin) by a mixed anhydride technique.
3. Incorporation of hapten into protein can be measured by phosphorus analysis.
4. ^{31}P-NMR indicates the hapten is intact in the resulting conjugates.
5. The conjugation reaction is reproducible and the molar incorporation ratio is a function of the reactant ratio. For bovine serum albumin this function is linear up to an incorporation ratio of at least 25:1.
6. Syntheses to date have involved diastereoisomeric mixtures of the soman analogs, but the techniques used would be applicable to synthesis of isomerically pure analogs.

^{31}P-NMR of Phosphonates



ANTIBODY SPECIFICITIES GENERATED AGAINST TWO ORGANOPHOSPHATE HAPTENS

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O.H.S.U., Portland, Oregon

We are studying the combining site specificities of monoclonal antibodies that we have raised against the two haptens, Soman (pinacolylmethylphosphonofluoridate) and PC (phosphocholine). Both molecules are organophosphates and share some structural and spatial characteristics. There are, however, differences in their chemical structures which prevent apparent cross-reactivity between antibodies specific for Soman and PC.

The immune response to PC is characterized by the presence of two major groups of antibodies. Group I and Group II antibodies are differentiated on the basis of their specificity for free PC or for a nitrophenyl derivative of PC. Interestingly, we have detected Group I-like and Group II-like antibodies in the immune response to Soman, i.e. they show differential specificity for free Soman and for nitro- and aminophenyl derivatives of Soman.

Introduction

Soman is a neurotoxin which acts on the acetylcholinesterase system. PC is a common environmental antigen found in the membrane of many bacteria, fungi, and mammalian cells. Both haptens are organophosphates which share limited spatial and structural properties, and are coupled to protein carriers via a diazophenyl linkage. Highly specific antibodies are raised in response to Soman and PC as evidenced by the lack of immunologic cross-reactivity between antibodies raised against the two molecules.

It was of interest to determine the fine specificity of the antibody combining sites of our Soman-specific monoclonals. In the course of characterizing these specificities, it was found that, similar to the PC system, anti-Soman monoclonal antibodies could be divided into two groups based on their ability to be inhibited by certain derivatives of Soman.

Implications of the work described here, comparing the specificities of antibodies raised against Soman and PC, are discussed as well as the use of this system in studying the generation of antibody diversity at the genetic level.

Materials and Methods

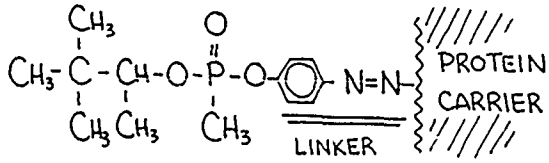
The purification and characterization of PC-specific monoclonal antibodies has been described previously (S.P. Chang et. al., *J. Immunol.* 132:1550, 1984).

Monoclonal antibodies against Soman were prepared after longterm immunization of BALB/c mice with Soman conjugated to keyhole limpet hemocyanin (So-KLH). Spleen cells were removed four days after boosting with So-KLH and fused with the mouse myeloma line FO. Hybridomas were screened for the production of anti-Soman antibodies in an enzyme-linked immunosorbent assay (ELISA) using Soman-conjugated bovine serum albumin (So-BSA) as test antigen. The hybridomas were cloned by hanging drop culture and the isotypes of the monoclonal antibodies determined with heavy chain specific antibodies. Anti-Soman antibodies were purified by affinity chromatography on Soman-glycyltyrosine-Sepharose 4B and eluted with 3M NaSCN, pH 8.0.

Fine specificity analysis of the hybridoma antibodies was carried out using an ELISA inhibition assay in which the ability of Soman or PC analogs to inhibit the binding of antibody to Soman-BSA was tested. The inhibitors included dipinacolylmethylphosphonate (DPMP), diisopropylmethylphosphonate (DIMP), p-aminophenyl-Soman (p-NH2-Ø-So), p-nitrophenyl-Soman (p-NO2-Ø-So), hydroxy-Soman (OH-So), phosphocholine (PC), p-nitrophenylphosphocholine (NPPC), So-BSA, and PC-BSA. I_{50} values were calculated by determining the millimolar concentration of free hapten that would cause 50% inhibition of binding.

SOMAN

Fig 1A



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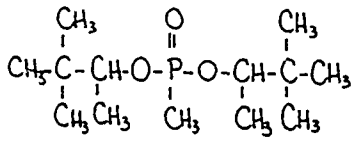
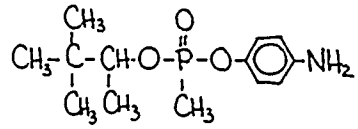
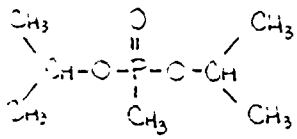


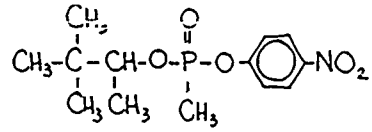
DIPINACOLYLMETHYLPHOSPHONATE DPMP



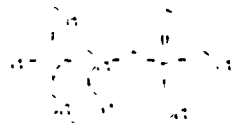
p-AMINOPHENYL-SOMAN $PNH_2\phi So$



DIISOPROPYLMETHYLPHOSPHONATE DIMP



p-NITROPHENYL-SOMAN $PNO_2\phi So$



HYDROXY-SOMAN OH So

Fig 1. (a) Chemical structure of Soman when linked to a protein carrier. The Soman derivatives used as inhibitors include DPMP, DIMP, p-NH2-ϕ-So, p-NO2-ϕ-So, and OH-So.

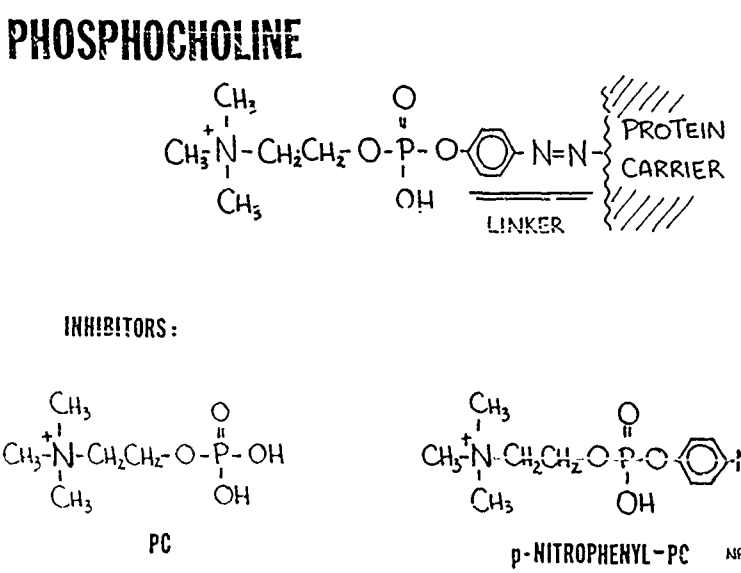


Fig. 1. (b) Chemical structure of PC when linked to a protein carrier. PC analogs used as inhibitors in the ELISA are free PC hapten and NPPC.

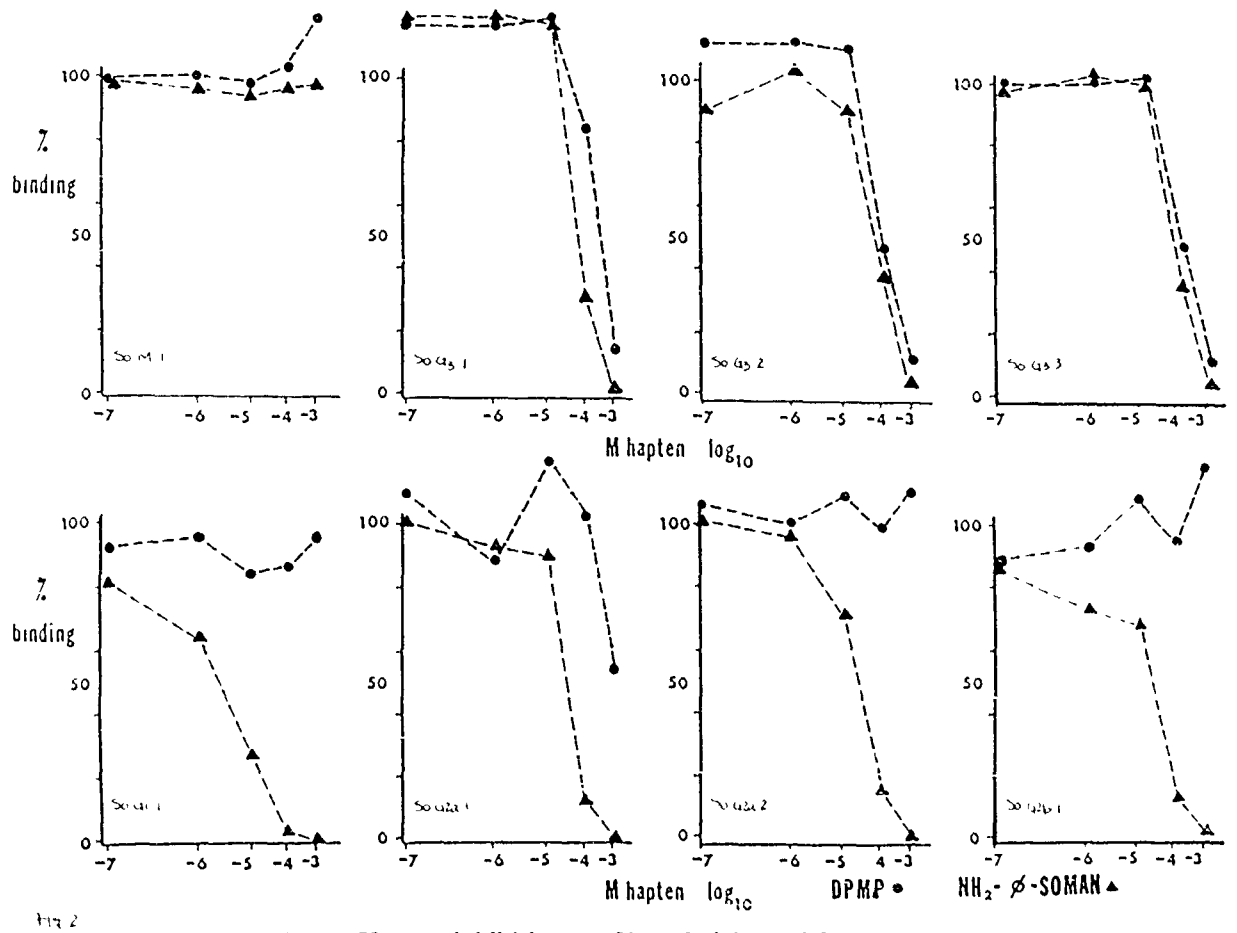


Fig. 2. Hapten inhibition profiles of eight anti-Soman antibodies using DPMP and p-NH2-ø-So as inhibitors. Group I antibodies (So-G3-1, So-G3-2, So-G3-3) are inhibited equally well by both inhibitors. Group II antibodies (So-G1-1, So-G2a-1, So-G2a-2, So-G2b-1) show greater inhibition by p-NH2-ø-So than by DPMP.

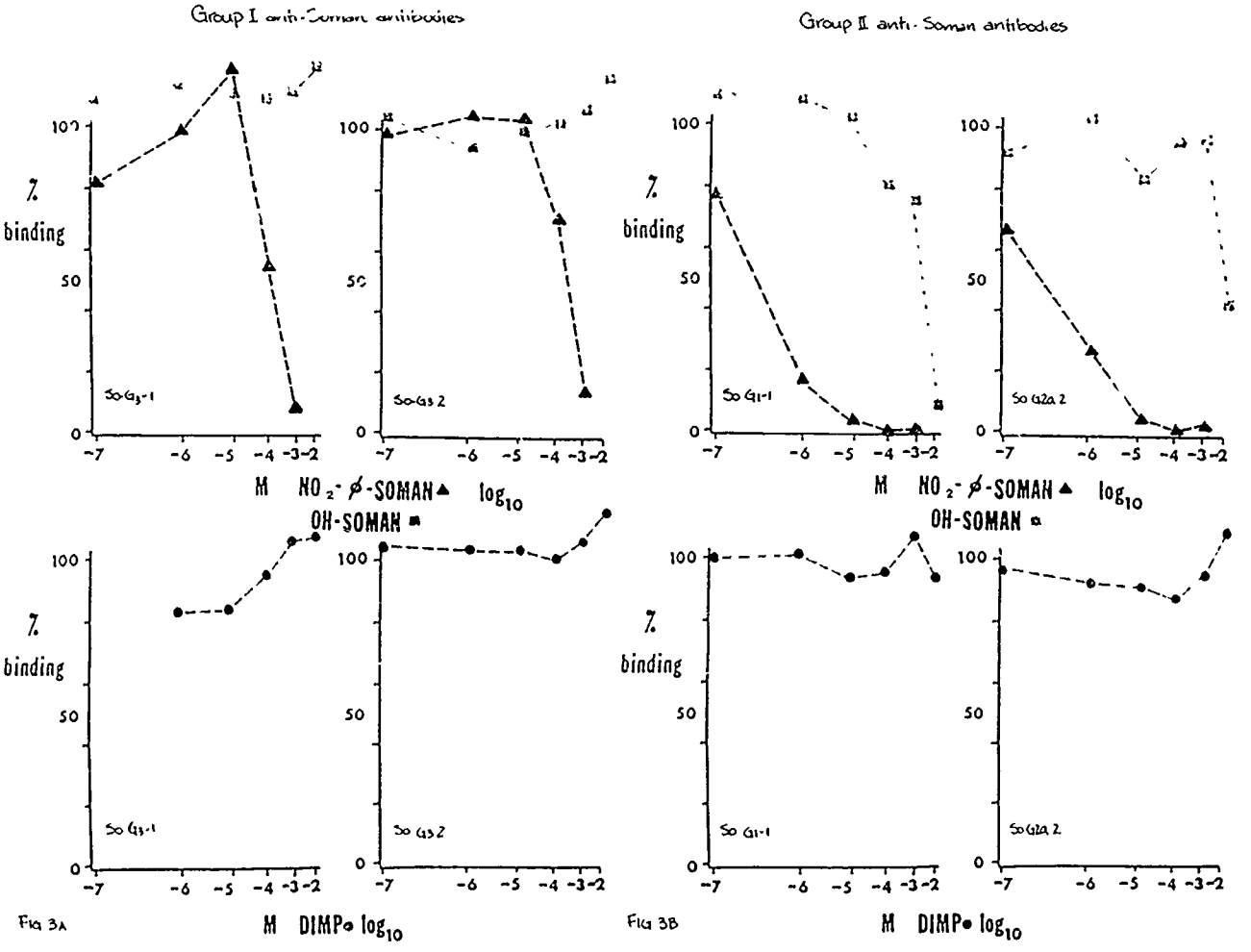


Fig. 3 Hapten inhibition profiles of representative Group I and Group II antibodies using DIMP, p-NO2-Ø-So, and OH-So as inhibitors. **(a)** Group I antibodies are inhibited by p-NO2-Ø-So, but not by OH-So or DIMP. **(b)** Group II antibodies are inhibited by p-NO2-Ø-So and only at high concentrations by OH-So. No inhibition is seen with DIMP.

TABLE I I_{50} VALUES (mM) FOR SOMAN - SPECIFIC MONOCLONAL ANTIBODIES

	inhibitors		ratio	inhibitors:		
	DPMP	p-NH_2-Ø-So	$\frac{I_{50}\ NH_2 Ø So}{I_{50}\ DPMP}$	DIMP	p-NO_2 Ø-So	OH-So
GROUP I						
So-G_3-1	0 315	0 062	0 2	>10	0 127	>10
So-G_3 2	0 090	0 059	0.65	>10	0 234	>10
So G_3-3	0 092	0.060	0 65			
GROUP II						
So-G_1 1	>1	0 002	<0 002	>10	0 00029	2.49
So G_{2a}-1	1	0 033	0.033			
So-G_{2a}-2	>1	0 018	<0 018	>10	0 00026	7 26
So-G_{2b}-1	>1	0 022	<0 022			
So M-1	>1	>1	—			

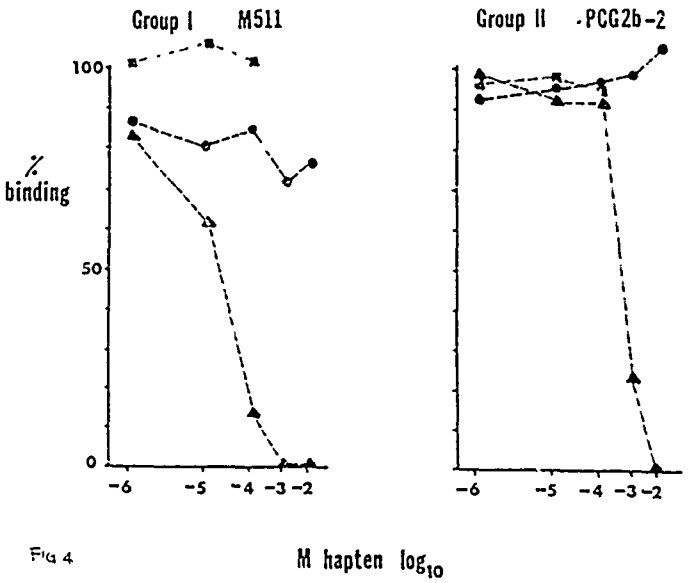


Fig 4

Fig. 4 (a)(b) Antibodies binding to PC-Histone are not inhibited by the presence of Soman-BSA (▪) or the Soman hapten DPMP (●). Shown as a positive control is inhibition of both M511 and PCG2b-2 by the PC analog NPPC (▲).

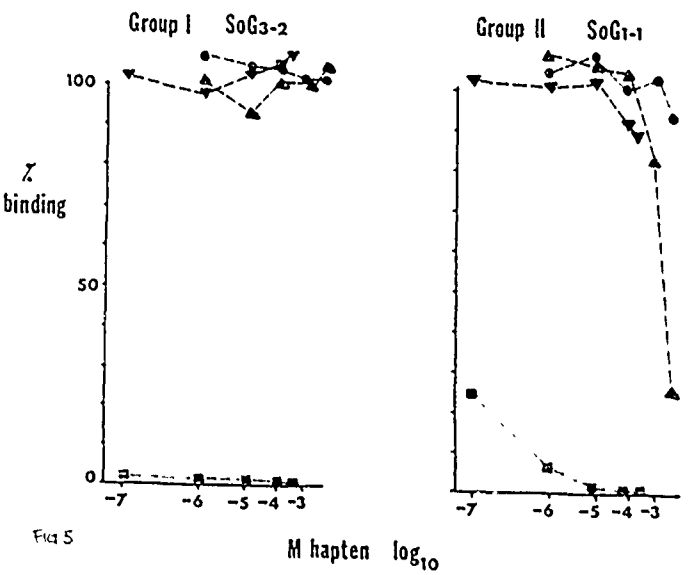


Fig 5

Fig. 5 (a)(b) Antibodies binding to So-BSA are not inhibited by the presence of PC-BSA (▼) or by free PC hapten (●). However, SoG1-1, but not SoG3-2, appears to be significantly inhibited by NPPC (▲). Very efficient inhibition by Soman-BSA (▪) provides a positive control.

Results

(1) Antibodies raised against Soman and PC are not cross-reactive for the other. The fine specificity analysis of anti-Soman hybridoma antibodies reveals two major groups distinguished on the basis of differential inhibition by Soman analogs. Group I antibodies are inhibited equally well by DPMP, p-NH2-Ø-So, and p-NO2-Ø-So. Group II antibodies are inhibited significantly only by p-NH2-Ø-So and p-NO2-Ø-So. Group II antibodies can also be inhibited by higher concentrations of OH-So. No Soman-specific antibody was inhibitable by the analog DIMP.

(2) Both Soman- and PC-specific antibodies appear to recognize at least two different regions of their cognate hapten molecules:

   (a) Group I antibodies appear to be directed against the region(s) involving the pinacolyl moiety for Soman and the choline moiety for PC, based on their inhibition by DPMP and PC, respectively.

   (b) Group II antibodies have specificity more heavily involving the phenylphosphate regions of the haptens. However, if these regions alone accounted for binding, we might expect NPPC to inhibit anti-Soman antibodies and p-NH2-Ø-So to inhibit anti-PC antibodies.

(3) So-G1-1, an anti-Soman monoclonal which is in Group II, represents an interesting case since it appears to be inhibitable by NPPC, the PC analog which characteristically inhibits Group II anti-PC antibodies. Thus, this monoclonal could represent an antibody in which recognition is directed primarily at the phenylphosphate region.

(4) So-M-1 is an IgM monoclonal antibody which is not inhibited by either DPMP or p-NH2-Ø-So, and therefore cannot be considered a Group I or Group II antibody. However, it is known to be specific for Soman because it binds Soman-BSA but not PC-BSA. One explanation for the inability to inhibit So-M-1 with Soman haptens may be that So-M-1 is a low affinity antibody. If this is the case, So-M-1 may represent a protein which corresponds to the germline sequences of the variable region genes used in the immune response to Soman.

(5) The distribution of monoclonal antibodies into Group I and Group II in both the Soman and PC systems correlates with the pattern of isotype distribution in each group:

(a) Group I antibodies are dominated by IgM, IgA, and IgG3 isotypes in the PC system and, consistent with this, are associated only with the IgG3 isotype in the Soman system.

(b) Group II antibodies have a strong preference for the IgG1, IgG2a, and IgG2b isotypes in both the Soman and PC systems.

Conclusions

(1) We have raised highly specific monoclonal antibodies against the two haptens, Soman and PC. It appears that charge plays a key role in the binding of antibodies to PC and hydrophobic functional groups are important in the binding of anti-Soman antibodies. A similarity in spatial orientation is not sufficient to cause cross-reactivity between the two types of antibodies.

(2) The presence of Group I and Group II antibodies in the Soman immune response, as detected by fine specificity analysis, indicates that there are at least two different immunogenic regions presented by the immunizing form of Soman. Group II antibodies characteristically bind phosphophenyl derivatives of Soman much better than the Soman analog DPMP. The finding that Group I and Group II antibodies appear to follow a common pattern of isotype distribution suggests that specific isotypes may be correlated with the generation of antibodies with affinity for phosphophenyl linkages.

(3) Since the Group II antibodies appear to be directed against regions containing the phenylphosphate structure in both PC and Soman, there exists a possibility that these antibodies utilize the same or related variable region heavy (VH) and/or light (VL) chain genes. If this is indeed the case, the Soman/PC system would be an excellent model system in which to study the relationship of particular DNA sequences to the fine specificity expressed by the complete antibody molecule.

SECRETION OF "MONOCLONAL" ACETYLCHOLINESTERASE BY A RAT HEPATOMA X MOUSE HEPATOCYTE HYBRID

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Department of Pediatrics, Uniformed Services University, Bethesda, MD

ABSTRACT

Primary cultures of mouse hepatocytes were fused with HGPRT-deficient, chemically transformed rat liver cells and hybrid cells were selected on the basis of their ability to grow in HAT medium. Cell cultures were assayed for secretion of cholinesterase by the hydrolysis of choline esters; most cultures exhibited little or no activity. However, one hybrid culture (E-2) was identified which secreted an enzyme capable of hydrolyzing acetylthiocholine and proprionylthiocholine, with little activity towards butyrylthiocholine. The enzyme was inhibited by soman, eserine sulfate and BW28451, but not by iso-OMPA, strongly suggesting that the enzyme is a form of acetylcholinesterase (E.C. 3.1.1.7). As neither of the parental cells secretes this enzyme in vitro, the E-2 hydrid may contain a chromosomal rearrangement that allows expression of a silent (possibly fetal) acetylcholinesterase gene. The hybrid cell line has a modal chromosome number of 56 which is different from that of both the parental cell types. The hybrid has a generation time of less than 24 hours and has been in continous culture for over 14 months, during which time the exprcssion of the acetylcholinesterase activity has remained constant. The enzyme is collected by culturing the E-2 cells in serum free medium; growth in such a medium should provide an excellent starting material for purification procedures. The secreted enzyme does not interact with the non-ionic detergent, Triton-X 100. On the other hand, the activity of cellular homogenates is much greater when extracted into detergent-containing buffer, suggesting that some of the intracellular enzyme may be membrane bound. Thermolability studies show that the secreted enzyme has characteristics of the tetrameric G_4 form of the enzyme: no inactivation at 48°C and approximately 50% inactivation at 54°C. Molecular weight studies (gel filtration, PAGE) show that the acetylcholinesterase is 320,000 daltons, consistent with the known size of the G_4 molecule. Because of these characteristics, these cells may be useful as a continuous source of enzyme for organophosphate diagnostic and therapeutic applications.

This work supported in part by the U.S. Army Medical Research and Development Command and the U.S. Army Armaments, Munitions and Chemical Command.

MICRO-ELLMAN REACTION

Reagent	Rxn 1	Rxn 2
Buffer	140ml	170ml
DTNB (3.3×10^{-4}M)	30ml	30ml
ATCI (5×10^{-3}M)	30ml	0ml
Cell Supernatant	100ml	100ml
	300ml	300ml

Abs 414 (Rxn 1) - Abs 414 (Rxn 2) = ΔA

CHOLINESTERASE ACTIVITY OF E-2 SUPERNATANT

1. Cells are grown in Williams Medium E (WME) with 10% FBS, 2mM L-glutamine and 10mM HEPES.

2. To collect enzyme, cells are washed 2x in serum-free WME and refed with 8-10ml of serum-free WME without phenol red.

3. Supernatants are collected 18 hrs later, centrifuged and tested.

Sample		Δ A	mU/ml[b]
E 2 supernatant	(1)	0.280	7.2
	(2)	0.265	6.6
AChE	8.0[a]	0.882	
	4.0	0.458	
	2.0	0.248	
	1.0	0.133	
	0.0	0.020	

[a] Electric Eel AChE, mU/ml
[b] Corrected for dilution

THE EFFECT OF SUBSTRATES ON ΔA BY E-2 SUPERNATANTS

Substrate	ΔA	
	Without Supernatant	With Supernatant
ATCI	0.038	0.355
PTCI	0.027	0.200
BTCI	0.018	0.007

All substrates were used at a final concentration of 0.5×10^{-3} M

SECRETION OF CHOLINESTERASE BY PARENTAL CELL CULTURES[a]

Supernatant	Inhibitor[b]	ΔA[c]
FRL	-	0.03
	B	0.04
	I	0.03
Hepatocytes	-	0.33
	B	0.32
	I	0.04

[a]30 minute assay, ATCI substrate

[b]B = BW284C51 (2×10^{-6}M); I = iso-OMPA (5×10^{-6}M)

[c]spontaneous hydrolysis: ΔA = 0.05

EFFECT OF HEAT TREATMENT ON CHOLINESTERASE IN E-2 SUPERNATANTS

Condition	mU/ml	Relative Activity
Control	3.03	100%
47°C/10 min	2.61	86.1
55°C/10 min	1.59	52.5
62°C/10 min	0.33	10.9

EFFECT OF INHIBITORS ON CHOLINESTERASE FROM E-2 HYBRID CELLS

		ΔA		
Inhibitor (M)	Substrate[a]	With Inhibitor	Without Inhibitor	Spontaneous
Eserine (1×10^{-5})	PTCI	0.007	0.280	0.023
Iso-OMPA (1×10^{-5})	PTCI	0.197	0.193	0.040
Paraoxon (5×10^{-6})	PTCI	0.053	0.200	0.058
BW284C51 (2×10^{-6})	ATCI	0.027	0.453	0.037

[a]PTCI = proprionylthiocholine iodide; ATCI = acetylthiocholine iodide

SECRETION OF ACHE BY CELLS GROWN IN CHOLINESTERASE FREE FBS

1. Serum passed through 100,000 m.w. filter to remove cholinesterase (CF-FBS)
2. Cultures switched to medium with CF-FBS on day 3 (~ 90% confluent)
3. Cultures refed with CF-FBS containing medium on day 4
4. Cultures refed with serum-free medium on day 5
5. 18 hours later, supernatants collected for AChE assay

Culture Condition	Δ A	mU/ml	mg/ml	mU/mg
10% FBS	0.280	7.20	1.13	6.37
10% CF-FBS	0.163	3.75	0.72	5.21
10% CF-FBS +20mg/ml EGF	0.273	6.90	1.42	4.86
20% CF-FBS, no cells	0.020	< 0	ND	ND

EFFECT OF TRITON X-100 ON ACHE ACTIVITY

Sample	TX-100	mU/ml	mU/mg
Supernatant	-	14.0	15.0
	+	13.5	14.4
Homogenate	-	13.2	7.4
	+	80.0	44.9

6. Pharmacokinetics and Distribution

THE USAMBRDL CHEMICAL AGENT HEALTH EFFECTS RESEARCH DIVISION

LTC W.R. Hartley, Chief, Health Effects Research Division
US Army Medical Bioengineering Research and Development Laboratory
Fort Detrick, Frederick, Maryland 21701

THE PRIMARY OBJECTIVE OF THE HEALTH EFFECTS RESEARCH PROGRAM AT THE US ARMY MEDICAL BIOENGINEERING RESEARCH AND DEVELOPMENT LABORATORY IS TO DETERMINE OR PREDICT THE POTENTIAL ADVERSE HEALTH CONSEQUENCES FROM REPEATED EXPOSURE TO LOW CONCENTRATIONS OF CHEMICAL AGENTS. CURRENTLY THE RESEARCH PROGRAM INCLUDES AGENTS GB (TYPE I AND II), VX, GD, GA, HD, L AND BZ. ALL RESEARCH ON AGENTS IS CONDUCTED EXTRAMURALLY WHILE RESEARCH ON HYDROLYSIS PRODUCTS AND SIMULANTS OF AGENTS IS DONE IN-HOUSE. THE PROGRAM INVOLVING GB, VX, GD, GA, L AND HD INCLUDES ESTABLISHING A COMPREHENSIVE HEALTH EFFECTS DATA BASE. A BATTERY OF TOXICOLOGICAL TESTS SERVES TO PREDICT ADVERSE HEALTH CONSEQUENCES AS WELL AS ELUCIDATE THE BASIC MECHANISMS OF TOXICITY DUE TO LOW DOSE EXPOSURES. THIS REGIMEN OF TOXICOLOGICAL TESTS INCLUDES BIOLOGIC ENDPOINTS FOR MUTAGENICITY, DELAYED NEUROPATHY, TERATOLOGY, REPRODUCTION AND 90-DAY SUBCHRONIC EXPOSURE AND WILL BE USEFUL IN ASSESSING HEALTH RISK DUE TO OCCUPATIONAL, FIELD AND COMBAT EXPOSURES OF MILITARY PERSONNEL, DA CIVILIANS AND CONTRACT WORKERS. THE RESEARCH RESULTS WILL SIGNIFICANTLY CONTRIBUTE TO THE DATA BASES NECESSARY FOR STANDARD/CRITERIA SETTING TO PROTECT PERSONNEL. SPECIFIC APPLICATIONS INCLUDE FIELD TRAINING, PRODUCTION AND DEMILITARIZATION ACTIVITIES, GUARDING ACTIONS, AND DRINKING WATER QUALITY. RESEARCH ON NON-SURETY MATERIALS INCLUDES THE CHEMISTRY AND TOXICOLOGY OF WATER TREATED WITH HYPOCHLORITE TO DETOXIFY AGENT VX AND THE DEVELOPMENT OF A CONFIRMATORY CHEMICAL TEST IN URINE OR BLOOD FOR OCCUPATIONAL EXPOSURE TO BZ. OTHER RELATED PROJECTS INCLUDE EVALUATION OF ADSORBENTS FOR THE RECOVERY, ENRICHMENT AND TRANSPORT OF AGENTS FOUND IN WATER, REMOVAL OF CW AGENTS FROM FIELD WATER SUPPLIES BY REVERSE OSMOSIS (SIMULANTS ONLY) AND DEVELOPMENT OF ANALYTICAL REFERENCE STANDARDS OF HYDROLYSIS PRODUCTS OF CW AGENTS. THE CURRENT HEALTH EFFECTS RESEARCH PROGRAM WILL BE COMPLETED BY FY88. THE FURTHER EXPANSION OF THE PROGRAM WILL DEPEND ON THE NEED FOR HEALTH EFFECTS RESEARCH ON NEW AGENTS OR BREAKDOWN (METABOLIC) PRODUCTS OF CURRENT INVENTORY AGENTS. THE ULTIMATE GOAL IS TO PRODUCE COMPLETE HEALTH EFFECTS CRITERIA DOCUMENTS ON ALL CURRENT INVENTORY AGENTS BY FY88.

OBJECTIVES

To conduct toxicological research to develop a complete health effects data base on inventory chemical warfare agents.

To determine the basic mechanisms of toxicity in repeated exposures to low concentrations of agents as related to health consequences.

To investigate the adverse health effects from potential occupational field and combat exposures of military personnel, Army civilians and contract workers, and laboratory personnel.

To define potential health implications for field soldiers from contact with or consumption of chemical warfare agents and their major hydrolysis or treatment products in field water supplies.

To establish appropriate animal models for health risk assessments and performance decrement measurement.

To provide the data bases for standard and criteria setting to protect the health of personnel in field training, demilitarization activities, guarding, munitions production and research.

To complete research on the following agents:

GA, Tabun (dimethylphosphoramidocyanidic acid, ethyl ester)

GB, Sarin (methylphosphonofluoridic acid, 1-methylethyl ester)

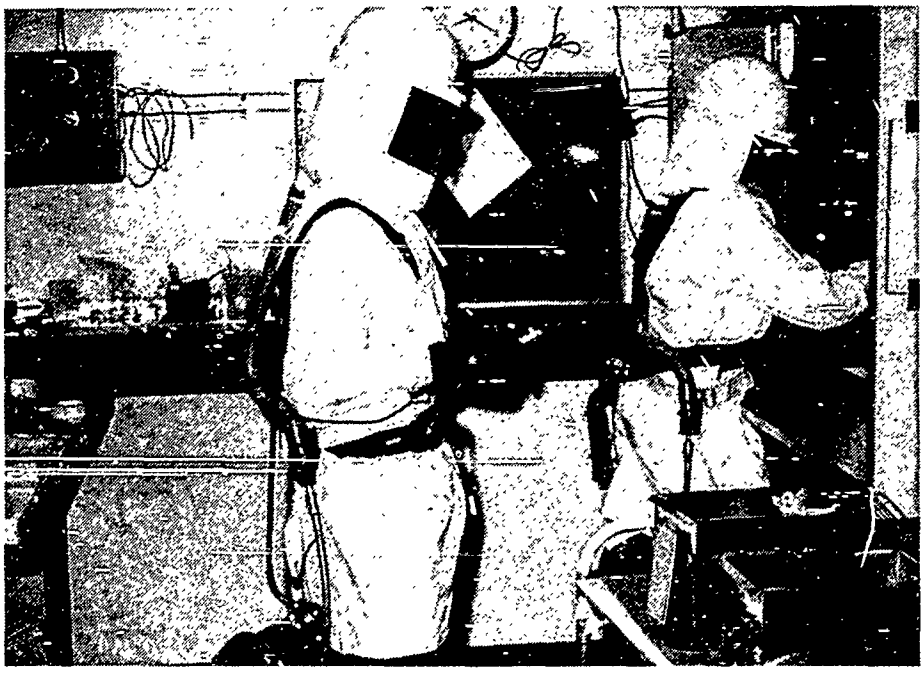
GD, Soman (methylphosphonofluoridic acid, 1,2,2-trimethylpropyl ester)

VX (methylphosphonothioic acid, S-[2- [bis(1-methylethyl)amino]ethyl] O-ethyl ester)

BZ (3-Quinuclidinyl Benzilate)

HD, Sulfur Mustard (1,1'-thiobis(2-chloroethane))

L, Lewisite (dichloro(2-chlorovinyl)arsine)



RATS INTUBATED WITH AGENT

SCIENTIFIC APPROACH

TOXICITY EVALUATION

Mutagenicity (in vitro selected tier tests):

A. Ames Salmonella Assay
   Five strains of Salmonella tested with and without S-9 metabolic activation
B. Saccharomyces Bioassay
   With and without S-9 metabolic activation
C. Mouse Lymphoma Forward Mutation Assay
   With and without S-9 metabolic activation
D. Chinese Hamster Ovary Cell Forward Mutation Assay
E. In Vitro Sister Chromatid Exchange Assay
F. Unscheduled DNA Synthesis in Rat Hepatocytes
G. Chromosome Aberration Assay
H. In Vivo Cytogenetics Assay in Rats

Delayed Neuropathy:

A. Single Dose in Chickens
B. Multiple Dose in Chickens

Teratology in Rats and Rabbits

Reproduction:

A. Two or Three Generation in Rats
B. Dominant Lethal (Modified) in Rats

90-Day Subchronic Oral in Rats

CHEMISTRY AND ENGINEERING EVALUATIONS

Sorption

Reverse Osmosis

Hydrolysis Product Organic Synthesis

EXTRAMURAL

US DEPARTMENT OF ENERGY
LABORATORY FOR ENERGY-RELATED HEALTH RESEARCH, UNIVERSITY OF CALIFORNIA
DAVIS, CA

P.I. M. GOLDMAN

Toxicity Studies on Agents VX, GB and GD

FOOD AND DRUG ADMINISTRATION
NATIONAL CENTER FOR TOXICOLOGICAL RESEARCH, JEFFERSON, AR

P.I. J. LaBORDE

Teratology Studies on Agents GB and GD

P.I. T. BUCCI

Toxicity Studies on Agents GB and GD

US DEPARTMENT OF ENERGY
BATTELLE PACIFIC NORTHWEST LABORATORIES, RICHLAND, WA

P.I. P. HACKETT

Teratology Studies on Lewisite and Sulfur Mustard Agents

P.I. L. SASSER

Toxicity Studies on Lewisite and Sulfur Mustard Agents

P.I. D. KALKWARF

Chemistry and Toxicology of Water Treated with Hypochlorite to Detoxify Chemical Agent VX

US NATIONAL BUREAU OF STANDARDS
NATIONAL MEASUREMENT LABORATORY, GAITHERSBURG, MD

P.I. E. WHITE

Development of a Confirmatory Chemical Test for Exposure to 3-Quinuclidinyl Benzilate (BZ)

DEPARTMENT OF ENERGY
LAWRENCE LIVERMORE NATIONAL LABORATORY, LIVERMORE, CA

P.I. L. ANSPAUGH

Data Base Assessment of Environmental and Toxicological Factors in Water to Upgrade and Modernize Content of TB MED 577.

IN-HOUSE

P.I. S. HOKE

Adsorbents for the Recovery, Enrichment or Transport of Chemical Warfare Agents Found in Water.

P.I. A. ROSENCRANCE

Analytical Reference Standards of Hydrolysis Products of CW Agents

P.I. W. BURROWS

Removal of Chemical Warfare Agents from Field Water Supplies by Reverse Osmosis: Development of Test Protocol and Efficacy Testing.

PRELIMINARY RESULTS

AGENT VX

MUTAGENESIS:

A. Ames Mutagenesis Assay - Negative tested in 5 strains of Salmonella (TA 98, TA 100, TA 1535, TA 1537, TA 1538) both with and without S-9 metabolic activation. Concentrations of 0.01, 0.05, 2.5, 10 µg VX/plate tested. (Remsen et al., 1984).[1]

B. Saccharomyces Bioassay - Negative tested in S. cerevisiae (strain D-7) both with and without S-9 metabolic activation. Concentrations of 25, 50, 100 µg VX/mL. (Shifrine et al., 1983).[2]

C. Mouse Lymphoma Assay - Negative tested in mouse lymphoma cells L5178Y both with and without S-9 metabolic activation. Concentration range of 1 to 100 µg VX/mL. (Kawakami et al., 1984).[3]

DELAYED NEUROPATHY:

A. Single dose in chickens - Negative in atropine/2-PAM treated chickens injected sc with 10, 100, or 150 µg VX/kg BW and examined by behavioral tests. (Wilson et al., 1984).[4]

B. Multiple dose study in chickens in progress.

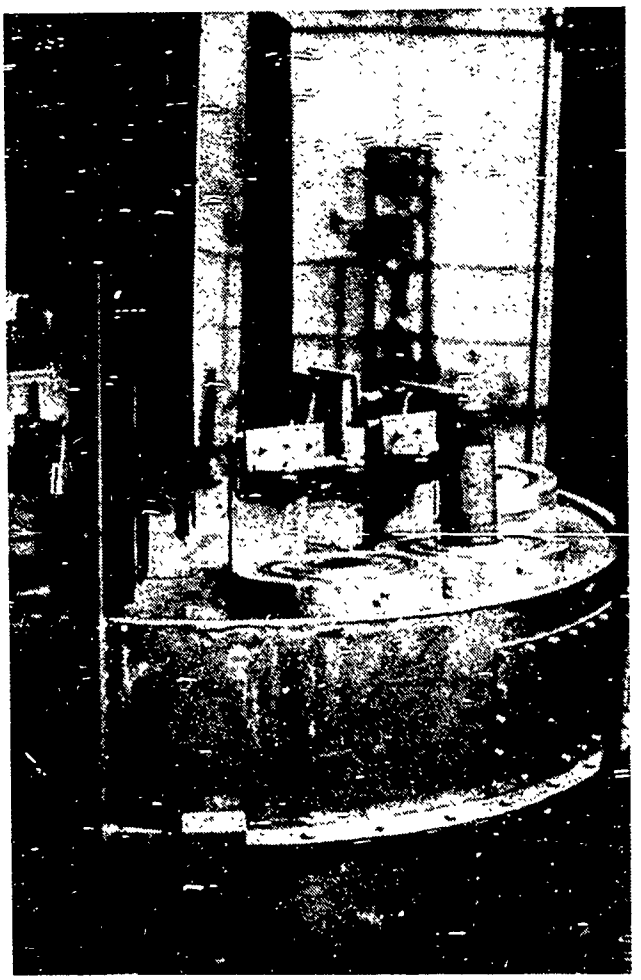
TERATOLOGY:

A. Negative in rats at dose levels of 0, 0.25, 1.0, and 4.0 µg VX/kg BW on days 6 through 15 of gestation. (Schreider et al., 1984).[5]

B. Teratology of VX in Rabbits - in progress.

REPRODUCTION:

A. Three Generation in Rats - in progress.



DEMILITARIZATION

B. Modified Dominant Lethal in Rats - in progress.

90-DAY SUBCHRONIC IN RATS: In progress.

AGENT GB

MUTAGENESIS: Ames Mutagenesis Assay, Mouse Lymphoma Assay, In Vitro Sister Chromatid Exchange Assay, and Unscheduled DNA Synthesis in Rat Hepatocytes - in progress.

DELAYED NEUROPATHY: Single and multiple dose studies in chickens in progress.

TERATOLOGY:

A. Negative in rats. GB Type I (tributylamine stabilizer) and GB Type II (Dicyclohexylcarbodiimide stabilizer), are not teratogenic when administered orally at doses of 100, 240 and 380 µg GB/kg BW. Maternal toxicity was noted in the 240 and 380 µg GB/kg groups for both Type I and II GB as evidenced by loss of body weight. (Lu et al., 1984).[6]

B. Teratology of GB in Rabbits - in progress.

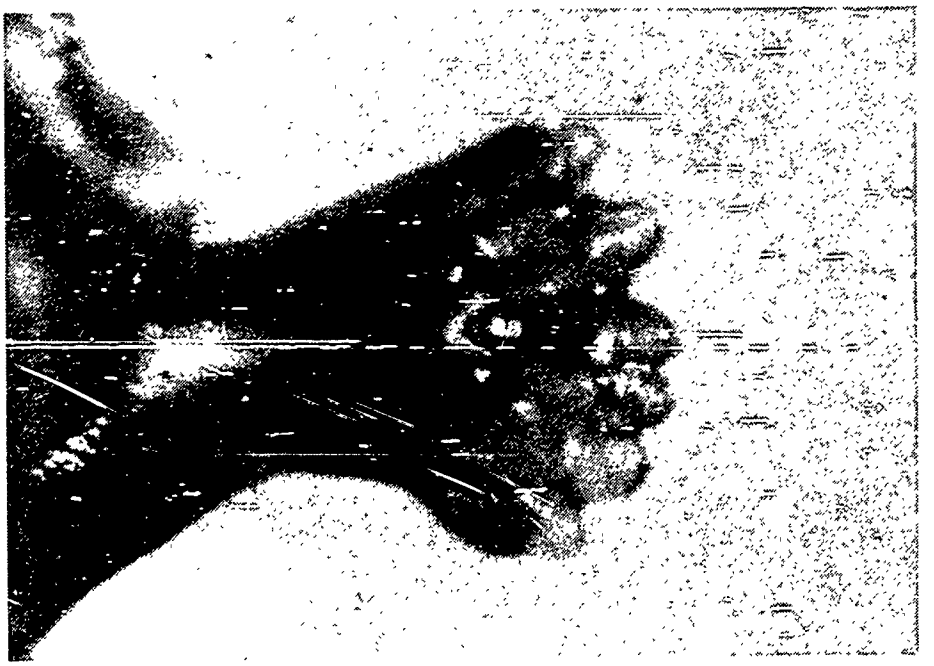
REPRODUCTION: Three Generation in Rats and Dominant Lethal in Rats - in progress.

90 DAY SUBCHRONIC IN RATS - in progress.

AGENT HD

TERATOLOGY:

A. Tested in pregnant rats at dose levels of 0.5, 1.0, 2.0 mg HD/kg BW on days 6 through 15 of gestation. Data analysis in progress. (Hackett, 1984).[7]



TERATOLOGY POLYDACTYLY IN RAT FETUS

B. Tested in pregnant rabbits at dose levels of 0.4, 0.6, 0.8 mg/kg BW of HD, and data are being analysed. (Hackett, 1985).[8]

REPRODUCTION AND 90-DAY SUBCHRONIC ORAL IN RATS: These studies will follow after the completion of the mutagenesis assays.

AGENT GD

Identical studies will be undertaken on GD as listed for GB. Range-finding studies for the rabbit teratology are in progress. Completion date is August 1985 for the mutagenesis studies and June 1987 for the remaining studies.

AGENTS GA AND L

Studies on mutagenesis, Delayed Neuropathy (GA only), Teratology, Reproduction and 90-Day Subchronic Oral In Rats will be started in FY86 and Completed by FY88.

AGENTS IN FIELD DRINKING WATER

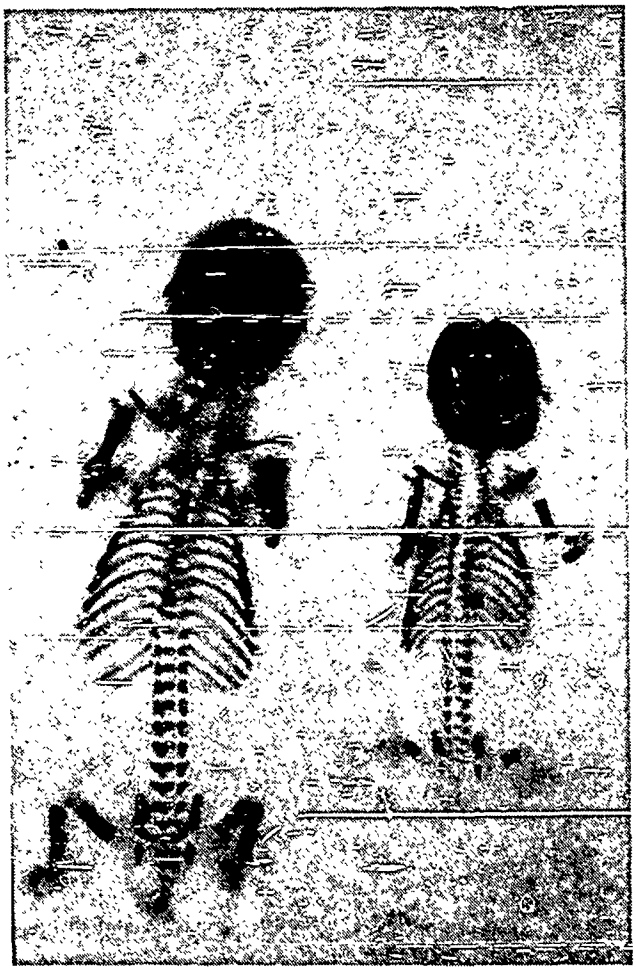
Interim field drinking water standards for chemical agents are being developed to address high water consumption rate (15L/day) in training or combat. FY86 completion.

AGENT DETECTION/CONCENTRATION

Agents GA, GB, VX and GD can be concentrated on adsorbent, eluted and detected with the enzyme ticket at levels less than 5 ppb, as opposed to the currently available detection level of 20 ppb. (Hoke and Shih, 1985).[9]

AGENT HYDROLYSIS PRODUCT SYNTHESIS

Currently have synthesized the alkyl alkylphosphonic acids for GB (1 kg), GA (400g) and VX (200g). Synthesis of GD hydrolysis product is in progress. (Rosencrance and Wade, 1985).[10]



TERATOLOGY

NORMAL & STUNTED RAT FETUS

CONCLUSIONS

THIS HEALTH EFFECTS RESEARCH EFFORT EVOLVED IN 1981 OUT OF A CONCERN FOR THE LACK OF TOXICOLOGY INFORMATION ON THE EFFECTS OF REPEATED, LOW DOSES OF AGENT. THE RELEVANCE OF THIS TYPE OF DATA IS OBVIOUSLY IN THE OCCUPATIONAL AND FIELD TRAINING/COMBAT ARENA. HOWEVER, IT SHOULD ALSO BE NOTED THAT THE TYPES OF STUDIES PRESENTED IN THIS RESEARCH PROGRAM ALSO PROVIDE BASIC RESEARCH INFORMATION ON LESS OBVIOUS ROUTES OF TOXICITY THAN STANDARD ACUTE MODES SUCH AS ANTICHOLINERGIC ACTIVITY. IT IS EXPECTED THAT THE COMPLETION OF THE EXISTING RESEARCH ON INVENTORY AGENTS (VX, GB, HD, L, GA, GD) WILL PROVIDE THE NECESSARY DATA TO PRODUCE MORE COMPREHENSIVE HEALTH EFFECTS CRITERIA DOCUMENTS IN FY87-88 THAT WILL BE USEFUL TO THE MEDICAL AUTHORITY IN ESTABLISHING STANDARDS TO PROTECT CIVILIAN AND SOLDIER HEALTH.

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TIME COURSE OF PHYSOSTIGMINE DISTRIBUTION, METABOLISM AND ChE INHIBITION IN RAT AFTER I.M. ADMINISTRATION

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Physostigmine (Ph) is one of the oldest anticholinesterase drugs which has a potential use as a prophylactic agent in the treatment of organophosphate intoxication. In order to study the time course of Ph in plasma, brain and other tissues, a sensitive method for the separation and determination of Ph and its metabolite was developed using high pressure liquid chromatography. The distribution and metabolism of 3H-Ph was studied in rat after i.m. administration (650 ug/kg). Four rats were decapitated at each time 2, 5, 7, 15, 22, 30, 60, 120 min and 24 hrs. Blood was collected. Brain and tissues were removed and frozen immediately until analyzed. Radioactivity (RA) was measured in tissues after combustion in the sample oxidizer. RA per gram of tissue peaked at 5 min except for liver and kidney. Peak concentration was highest in liver followed by kidney, adrenals, lungs, heart, plasma, spleen, brain, fat, muscle and eyes. Ratio of brain to plasma was about 0.9, 1.5 and 1.1 at 5, 30 and 60 min, respectively. Liver extensively metabolized Ph to eseroline, M1 and M2 metabolites. Metabolite M1 appears to be rubreserine, based on retention time on HPLC. The time course of 3H-Ph concentration and the ChE enzyme activity in plasma and brain was studied at the corresponding time. Plasma BuChE activity using the inhibitor BW284C51 and brain ChE activity were determined. Plasma, Ph concentration peaked at 5 min, .58 ug/ml, and decreased to .18 ug/ml at 22 min and .12 ug/ml at 30 min. Plasma BuChE activity was reduced to 60% of control activity in 2 min, 55% in 22 min, and 54% in 30 min. Ph plasma half-life was found to be 17

min and volume of distribution 1.28 liter/kg. Brain 3H-Ph concentration peaked at 5 min, .54 ug/g, and decreased to .26 ug/g at 22 min and .18 ug/g at 30 min. Brain ChE activity was reduced to 40% of control activity at 2 min, 22% at 5 min, 33% at 22 min, 40% at 30 min, and 63% at 60 min. Ph brain half-life was found to be 16 min. These results indicate that the inhibitory effect of Ph appears to diminish after 22 min in plasma and after 30 min in the brain. (Supported by U.S. Army Contract No. DAMD 17-83-C-3195.)

INTRODUCTION

Physostigmine (Phy), an alkaloid from the calabar bean, is a potent inhibitor of cholinesterase. It readily penetrates the central nervous system and has the potential as a prophylactic agent in the treatment of organophosphate intoxication. Recently, it has also been used in the treatment of Alzheimer patients.

Although the inhibition of plasma ChE by Phy was first studied as early as 1946 by Koster and then by Koelle in 1946, but to date, there is still no information available on the quantitation of Phy concentration and its relationship to ChE inhibition in plasma and brain after the administration of Phy to animals. The basic knowledge of distribution, metabolism and pharmacokinetics of Phy is still lacking.

We have studied the pharmacokinetics, tissue distribution, time course of metabolism of Phy in rat plasma, brain, and liver, and the relationship of Phy concentration to ChE inhibition in plasma and brain after i.m. administration.

METHODS

Male Sprague-Dawley rats weigning 200 to 300 g were deprived of food overnight and weighed. Each rat was administered 3H-Physostigmine (650 ug/kg body weight, i.m.). A group of four animals was killed by decapitation at 2, 5, 7, 15, 22, 30, 60 and 120 min after the dose. Blood was immediately collected in heparinized tubes, centrifuged at 2500 rpm for 20 min, plasma was separated and frozen until analysis. Liver, brain, adrenals, eyes, fat, heart, kidney, lung, muscle and spleen were removed from all animals, rinsed in ice-cold saline (0.9% w/v), blotted dry and stored at -70 C until analysis.

The radioactivity content in the tissue was measured by a Packard Tri-Carb Model 306 sample oxidizer. The 3H 0 from oxidation of tissue was collected in 15 ml of Monophase 40 Plus (Packard Instruments Co., Downers Grove, IL) liquid scintillation cocktail. The radioactivity was counted in a Beckman LS 5800 liquid scintillation spectrometer.

The time course of Phy metabolism was studied by determining drug and metabolites concentration in plasma, brain and liver using HPLC.

The butyrylesterase activity in plasma and ChE activity in brain were assayed according to a modification of the method of Johnson and Russell (1975). In this procedure, 3H-AChI (2 mmole per .1 uCi) was used as substrate. This method measures the radioactivity (RA) due to 3H-acetate formed by the enzymatic hydrolysis of 3H-acetylcholine. The inhibitor BW28C525 (1x10 M) is used with the plasma samples, so that only BuChE activity is reported. Blanks representing the nonenzymatic hydrolysis of acetylcholine were prepared with each batch of samples run and subtracted off as background. The specific activity of each freshly prepared batch of 3H-ACh was measured.

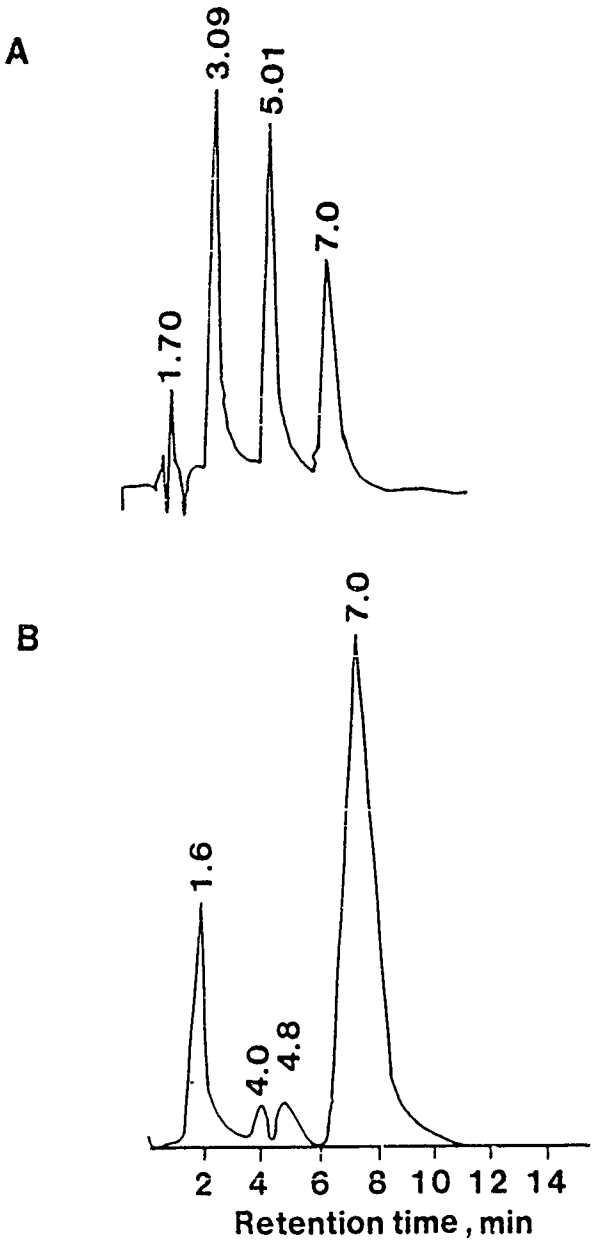
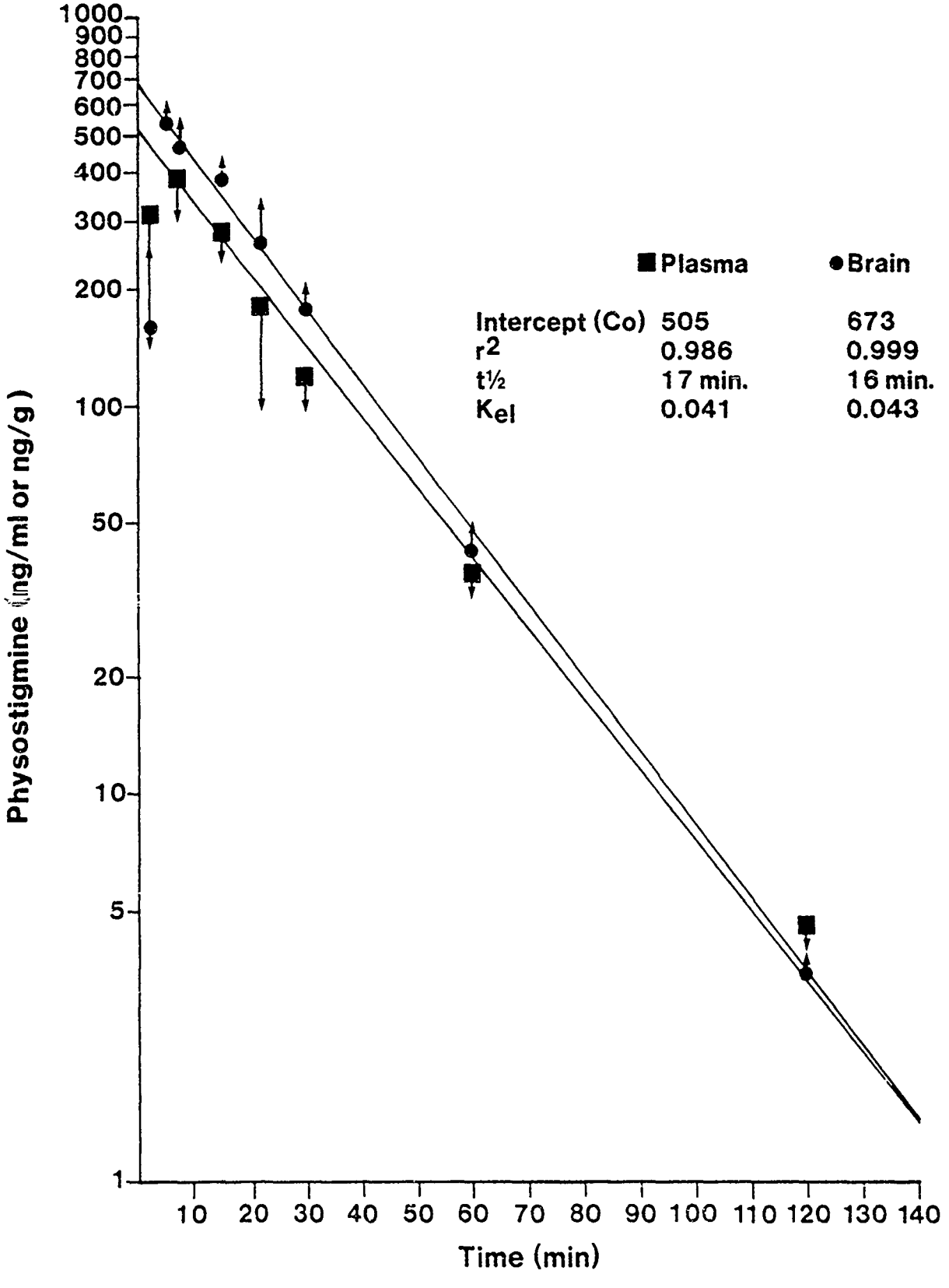


Figure A.
Separation of rubreserine (Rt 3.09), eseroline (Rt 5.01), and physostigmine (Rt 7.0) by HPLC using U.V. detection.

Figure B.
Separation of metabolites M_1 (Rt 1.6), M_2 (Rt 4.0), eseroline (Rt 4.8), and physostigmine (Rt 7.0) by HPLC, collecting radioactive fraction.

Pharmacokinetics of Physostigmine in plasma and brain



A-701

Concentration of physostigmine in plasma and brain and their ratio at various time intervals after 650 ug/kg i.m. administration.

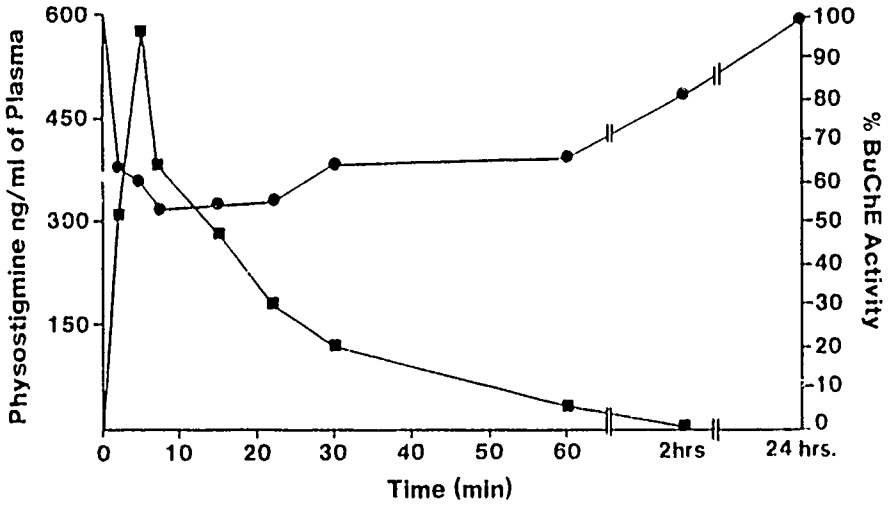
	Physostigmine		
Time (min)	Plasma (ng/ml)	Brain (ng/g)	Brain/Plasma
2	310.6 ± 160.6	157.1 ± 87.9	0.53 ± 0.15
5	582.9 ± 34.4	537.8 ± 51.7	0.93 ± 0.10
7	390.0 ± 75.5	463.3 ± 72.5	1.21 ± 0.23
15	278.7 ± 44.5	385.3 ± 31.9	1.40 ± 0.15
22	178.9 ± 101.3	264.8 ± 79.2	1.61 ± 0.32
30	120.5 ± 25.7	176.8 ± 26.4	1.49 ± 0.15
60	36.8 ± 4.7	42.0 ± 8.7	1.14 ± 0.17
120	4.5 ± 1.18	3.4 ± 1.97	0.74 ± 0.25

*Each value represents mean ± Std. Dev. of four rats.

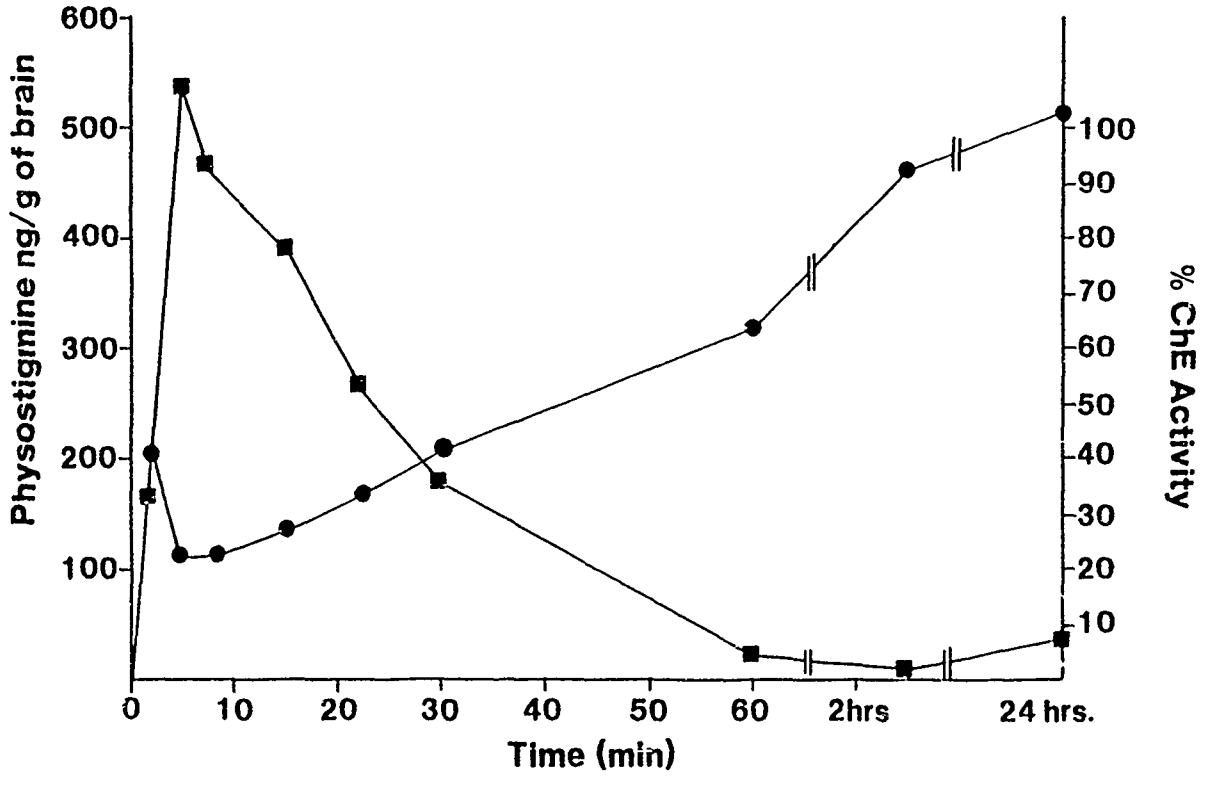
HPLC Conditions:

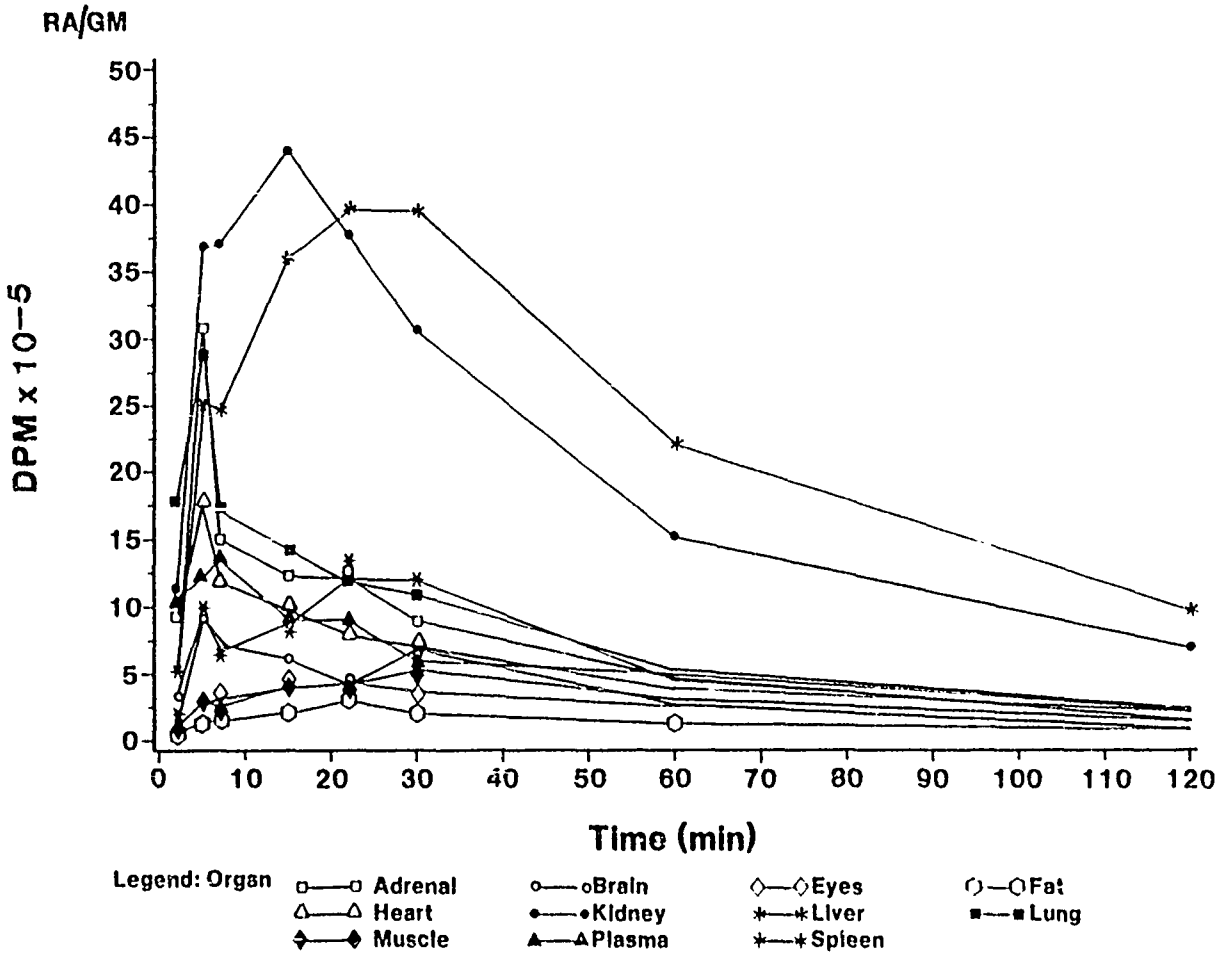
Column:	Waters C-18 u Bondapak 30 cm x 3.9 mm reversed phase.
Mobile Phase:	0.005 M Octanesulfonic acid + 0.005 M sodium phosphate monobasic + 1% v/v acetic acid in methanol:H_2O (40:60)
Flow Rate:	2 ml/min
Wavelength:	245 nm
Sample Loop:	100 ul
Detector:	Waters 481 variable wavelength LC spectrophotometer.

Time course of physostigmine (■) and BuChE activity (○) in plasma



Time course of physostigmine (■) and ChE activity (●) in brain

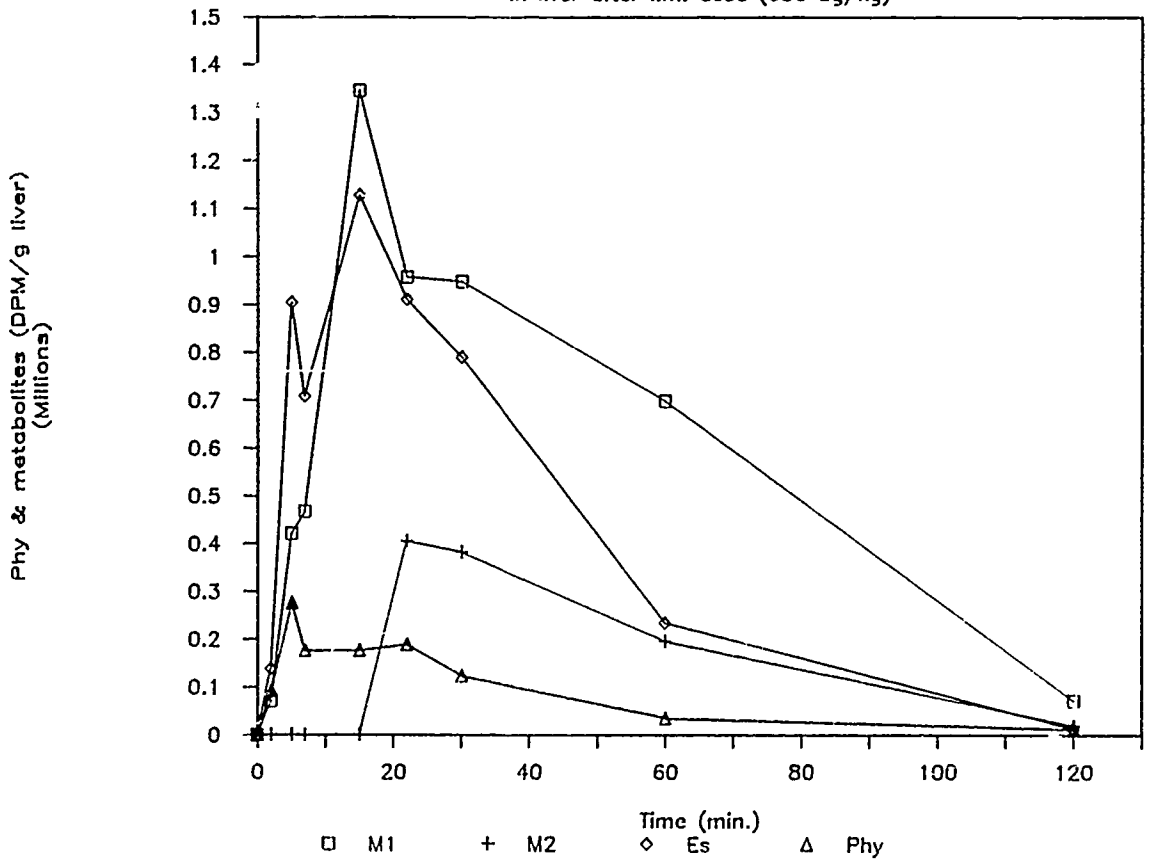




A-703

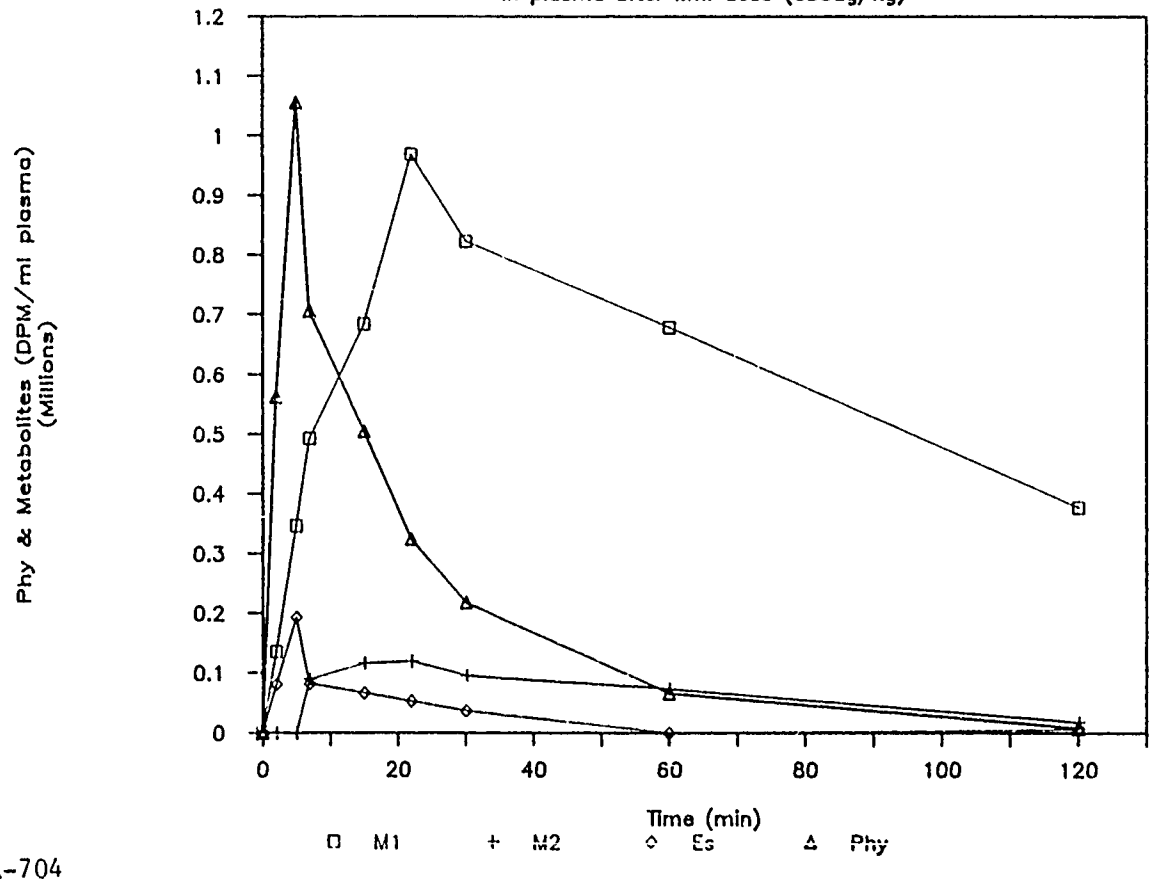
Time course of 3H–Phy & metabolites

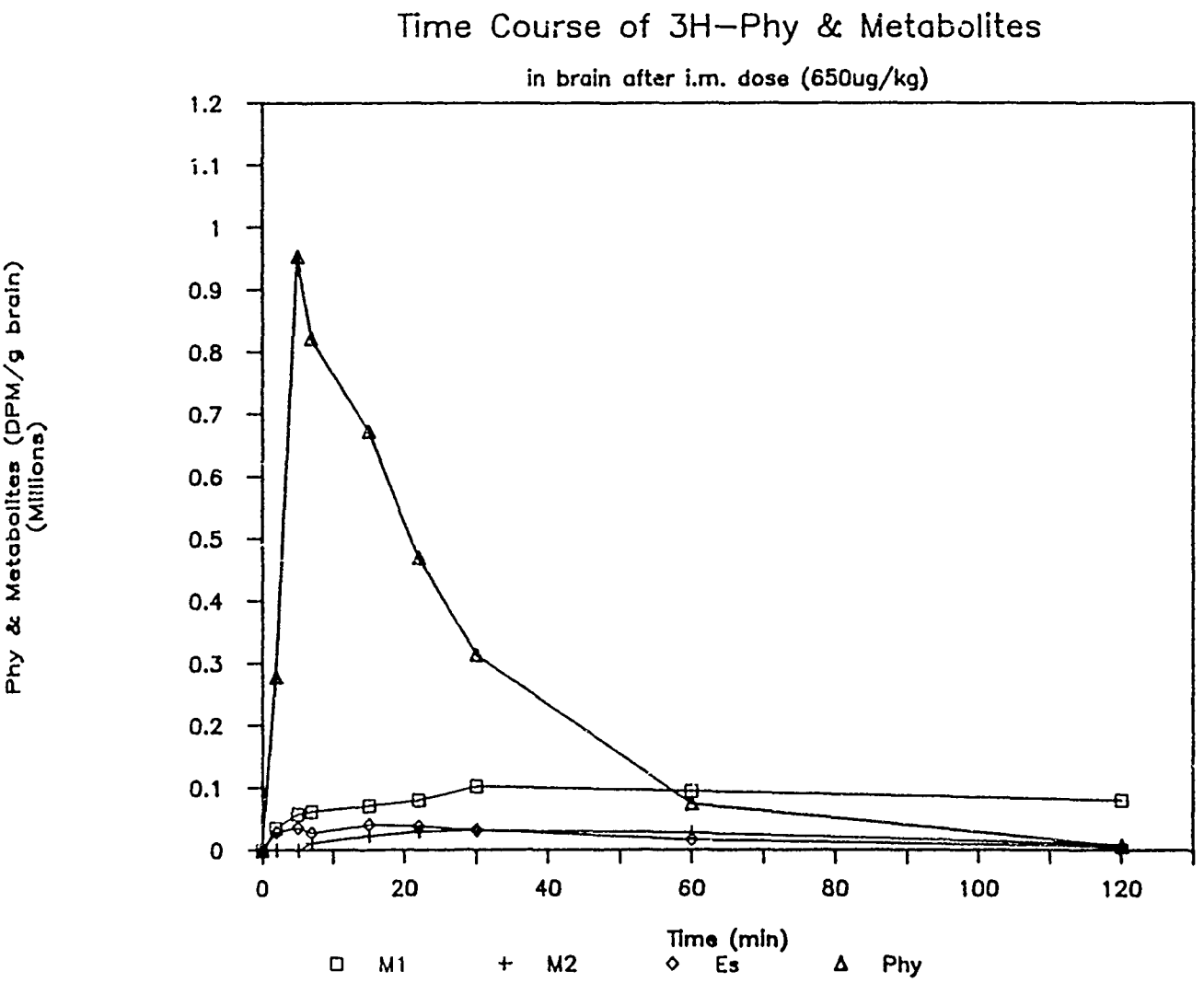
in liver after i.m. dose (650 ug/kg)



Time Course of 3H–Phy & Metabolites

in plasma after i.m. dose (650ug/kg)




Time Course of 3H–Phy & Metabolites
in brain after i.m. dose (650ug/kg)
Phy & Metabolites (DPM/g brain)
(Millions)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Time (min)
M1
M2
Es
Phy

SUMMARY

1) A high pressure liquid chromatographic method was developed for the resolution and determination of Phy and metabolites using UV detector and also by counting radioactivity in chromatographic fractions.
2) The half life of Phy in plasma, brain and liver was 17, 16 & 25 min respectively whereas the elimination rate constant was 0.041, 0.043 & 0.027 min^{-1} . The volume of distribution in plasma was 1.28 L/Kg and clearance was 52.6 ml min^{-1} kg^{-1} after i.m. dosage.
3) The radioactivity per gram tissue peaked at 5 min in adrenal, heart, lung & plasma. The peak occured at 15 min in kidney and at 30 min in liver, muscle and spleen. The percent of radioactivity administered was highest in muscle followed by liver, plasma, fat, kidney and other tissues.
4) Phy was metabolised to Eseroline and two other metabolites M1 and M2. The time course of Phy metabolism indicated that it was rapidly metabolised in plasma and liver. Only minute amounts of parent drug was present in plasma, brain and liver, 2 hours after administration. M1 appears to be a major metabolite.
5) The time course of BuChE activity in plasma showed immediate inhibition of activity. The maximum inhibition, 47% occured at about the same time (7 min) as peak plasma concentration (5 min). The enzymatic activity plateaued at about 65% from 30 to 60 min, recovered to 80% in 2 hr and 100% in 24 hr.
6) The time course of ChE activity in brain showed maximum inhibition (80 %) at the same time (5 min) as peak brain concentration. The enzymatic activity gradually recovered to 80 % in 2 hr and 100 % in 24 hr.

AUTORADIOGRAPHY SHOWS COMPARTMENTAL DISTRIBUTION OF 14-C (3,3-DIMETHYL-2-BUTOXY)-METHYLPHOSPHORYLFLUORIDE (SOMAN) IN THE CNS OF RATS

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ABSTRACT

The importance of acetylcholinesterase (AChE) inhibition as a mechanism of toxicity of organophosphorous (OP) poisoning has been shown by many investigators. However, long lasting effects of accidental exposure to OP compounds in humans seem to be unrelated to the immediate inhibition of AChE. These effects range from sensory motor polyneuropathies to sleep and memory disturbances, acute and chronic episodes of depression and severe anxiety attacks. The probable sites of action(s) and the mechanism of these disturbances remain obscure. The following study was undertaken to determine the time dependent distribution of soman in the central nervous system of the rat using quantitative autoradiography.

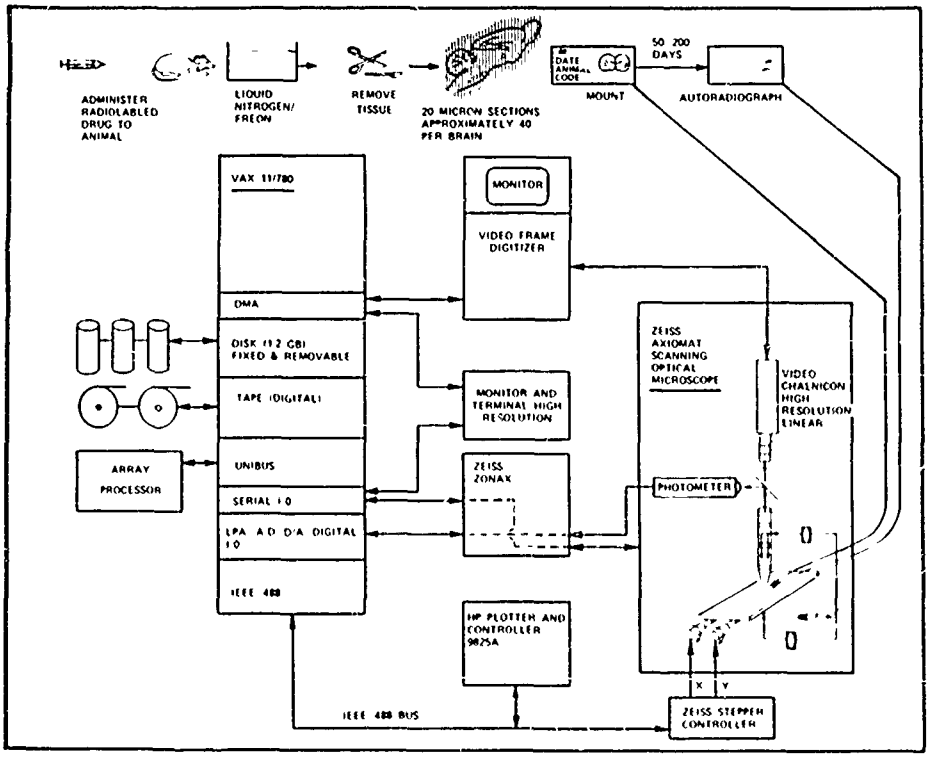
Radiolabeled soman (59 mCi/mmole) was administered in a single dose of 0.75 LD_{50} (17.8 ug/kg im) to 18 male Charles River rats. The animals were sacrificed at intervals of 2 and 32 minutes and 48 hours post exposure. Diffusional artifact of the radiolabeled drug was minimized by rapid freezing of the whole animal in Freon II at −80°C, extraction of the brain at −20°C, cryostatic sectioning and performance of autoradiography at −80°C. Autoradiographic exposures were made of 20 micron thick sections for periods of 50 to 300 days depending upon the signal strength in the areas of interest. The autoradiographs were measured using photometric methods which yielded both optical and area densities, grain counts and grain areas.

Results showed that the distribution of soman and/or its metabolites varied as a function of time. At 2 minutes, there was marked distribution to blood, choroid plexis and CSF, with small detectable differential accumulation occurring in the nucleus of the XII cranial nerve, area postrema, medial vestibular, prepositus hypoglossal and paragigantocellular reticular nuclei. At 32 minutes, the above compartments were relatively unchanged from the 2 minute observations and several additional areas were differentiated including the principal ocular and lateral posterior thalamic nuclei and cortical cell layers. At 48 hours, all the above labeling had been reduced to nearly background levels except for anteroventral thalamic, caudate, and accumbens nuclei. Both the caudate and accumbens showed higher labeling than any previously noted nuclei. The 48 hour accumulation and irreversible binding implies diffusional or actively limited molecular transport processes with subsequent covalent reaction. This may be due to slow movement of polar metabolites through the blood brain barrier which subsequently bind at sites in the caudate and accumbens nuclei. Because other sites of brain cholinesterase are not affected in a similar manner, the observed activity is probably not due to cholinesterase.

INTRODUCTION

MEDICAL, MILITARY, INDUSTRIAL AND AGRICULTURAL SIGNIFICANCE OF ORGANOPHOSPHOROUS (OP) COMPOUNDS AS POTENT DRUGS, INSECTICIDES AND POTENTIAL CHEMICAL WARFARE AGENTS HAS RESULTED IN EXTENSIVE INVESTIGATION OF THE ETIOLOGY OF OP POISONING. ALL SPECIES FROM DROSOPHILA TO HUMANS RESPOND WITH A VARIETY OF TOXIC SYMPTOMS TO VERY SMALL QUANTITIES OF OP COMPOUNDS. THE IMPORTANCE OF AChE INHIBITION AS A MECHANISM OF TOXICITY HAS BEEN ESTABLISHED BY A NUMBER OF INVESTIGATORS. HOWEVER, THE LONG LASTING EFFECTS SEEN IN HUMANS ACCIDENTALLY EXPOSED TO OP COMPOUNDS SEEM TO BE UNRELATED TO AChE INHIBITION. THESE INCLUDE SLEEP AND MEMORY DISTURBANCES, CHRONIC DEPRESSION AND/OR ANXIETY AND SENSORY MOTOR POLYNEUROPATHIES. ELECTROPHYSIOLOGICAL STUDIES PERFORMED ON SEVERAL PATIENTS ACCIDENTALLY POISONED WITH TRIORTHOCRESYLPHOSPHATE SHOWED AN IMPROVEMENT IN PERIPHERAL NERVOUS SYSTEM LESIONS AFTER 2-3 MONTHS WHILE LESIONS IN THE CENTRAL NERVOUS SYSTEM SHOWED LITTLE OR NO IMPROVEMENT AFTER YEARS OF OBSERVATION. QUESTIONS CONCERNING THE ACTUAL SITES OF ACTION, MECHANISMS OF ACTION, ROUTE OF METABOLITES, PHARMACOKINETICS OF DISTRIBUTION AND PHYSIOLOGICAL EFFECTS OF OP POISONING HAVE BEEN ASKED. A STUDY OF DFP BINDING USING HIGH RESOLUTION AUTORADIOGRAPHY IMPLICATED AT LEAST TWO MODES OF LESION FORMATION, ONE AT THE ACH RECEPTOR AND AChE SITES AND THE SECOND AT A MORE DIFFUSE SITE WHICH EVENTUALLY LED TO AXONAL DEGRADATION. AN ULTRASTRUCTURAL STUDY OF THE EFFECTS OF PAROXON HAS BEEN MADE AND INCLUDES CHANGES IN MUSCLE MYOFILAMENT ORGANIZATION, GOLGI SIZE AND SHAPE, VESICULAR FUSION PROCESS CHANGES, MUSCULAR Z BAND DISRUPTION, SUBSARCOLEMAL ABNORMALITIES AND VACUOLE FORMATION. THE SIGNIFICANCE OF OP POISONING MECHANISMS OTHER THAN THOSE WHICH RESULT IN EXCESS ACH ARE POORLY UNDERSTOOD AND RECENT STUDIES IMPLY AChE HAS FUNCTIONS BEYOND METABOLIZING ACH. THIS STUDY WAS INITIATED BECAUSE THE KINETICS OF SOMAN ABSORPTION, DISTRIBUTION, BINDING, METABOLISM AND ELIMINATION IN THE CENTRAL NERVOUS SYSTEM ARE NOT DEFINED AT LEVELS OF RESOLUTION CONSISTENT WITH THE SIZE OF THE FUNCTIONAL ENTITIES OF THE CENTRAL NERVOUS SYSTEM, NAMELY THE NUCLEI.

METHODS



RESULTS

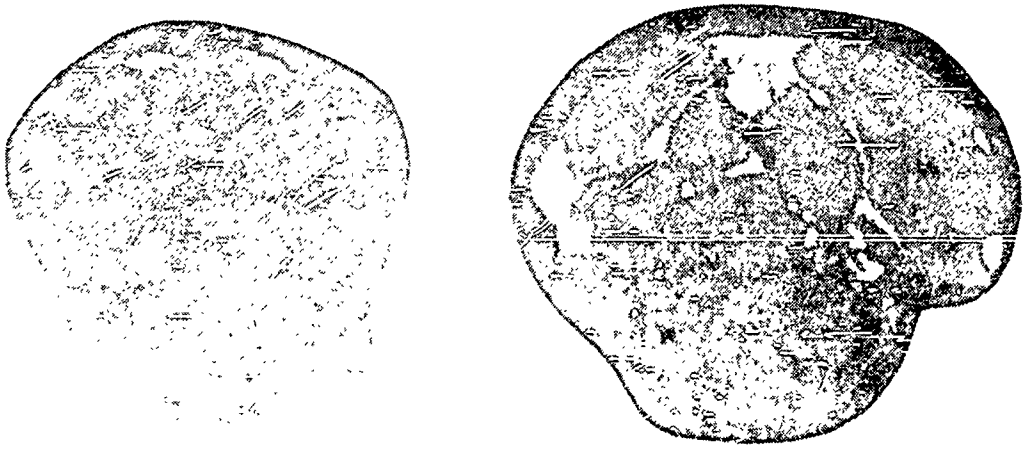
SOMAN WAS DISTRIBUTED TO AREAS OF THE RAT CNS LESS THAN 2 MIN AFTER AN IM INJECTION OF 17 MICROGRAMS/KG. AT 2 MIN, THE PRIMARY LOCATION OF THE RADIOLABEL WAS IN THE BLOOD, CSF AND CHOROID PLEXUS. THERE WERE SMALL BUT OBSERVABLE ACCUMULATIONS IN MOTOR XII NERVE, AREA POSTREMA, MEDIAL VESTIBULAR, PREPOSITUS HYPOGLOSSAL AND PARAGIGANTOCELLULAR RETICULAR NUCLEI. THERE WAS HOWEVER, GENERAL LOW LEVEL LABELING IN THE REMAINING BRAIN AREAS. AT 32 MIN, DIFFERENTIATION OF THE CELLULAR AND NEUROPIL AREAS OF THE NEOCORTEX AND HIPPOCAMPUS HAD BEGUN AND MINOR EMPHASIS IN THE PRINCIPAL OCULAR AND LATERAL POSTERIOR NUCLEI WERE NOTICED. THE 48 HOUR AUTORADIOGRAPHS SHOWED A MARKED ACCUMULATION IN THE CAUDATE AND ACCUMBENS NUCLEI WITH MINOR EMPHASIS IN THE SUPERIOR COLLICULUS AND ANTEROVENTRAL THALAMIC NUCLEI. ALL OTHER AREAS HAD REDUCED LABELING COMPARED TO THE CAUDATE AND ACCUMBENS NUCLEI. MYELINATED STRUCTURES, SUCH AS THE ANTERIOR COMMISSURE AND CORPUS COLLOSUM, UNIFORMLY CONTAINED THE LEAST LABEL IN ALL THREE EXPERIMENTAL PERIODS. THE HIGH LABELING INTENSITY IN THE CAUDATE AND ACCUMBENS NUCLEI ONLY OCCURED IN THE 48 HOUR TISSUES AND EXCEEDED ALL OTHER NUCLEI AT 2 MIN, 32 MIN AND 48 HOURS.

CONCLUSIONS

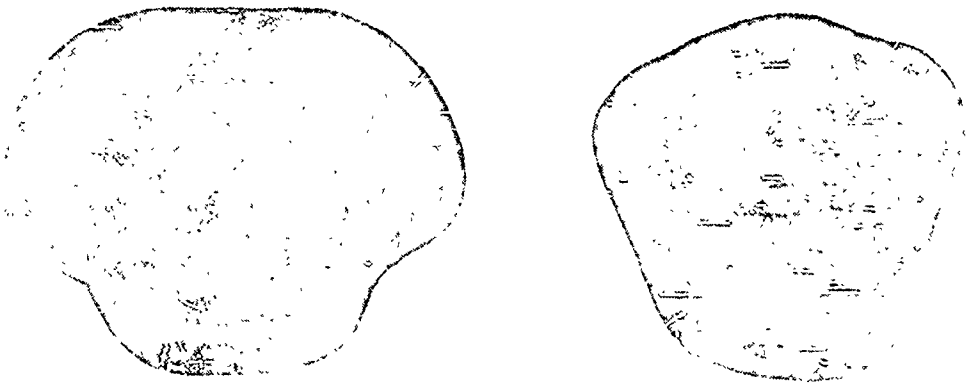
THERE ARE A CLASS OF REACTIVE SITES IN THE CAUDATE AND ACCUMBENS NUCLEI THAT ARE MORE SENSITIVE TO SOMAN AND/OR ITS METABOLITES THAN ANY OTHER CNS COMPARTMENT INCLUDING THOSE CONTAINING SIMILAR CONCENTRATIONS OF CHOLINESTERASE. THE FUNCTIONAL IMPLICATIONS OF THE LONG TERM RETENTION IN THESE NUCLEI ARE NOT CLEAR AT THIS TIME. IT IS ALSO NOT CLEAR WHETHER OR NOT THE OBSERVED RADIOLABEL IS SOMAN OR ITS METABOLITE(S). RECENT WORK WOULD IMPLY THAT IT IS THE AGED METABOLITE OF SOMAN, METHYLPHOSPHONIC ACID. A STUDY DIRECTED AT DETERMINING THE COMPOSITION OF THE ACCUMULATED LABEL HAS BEEN INITIATED.

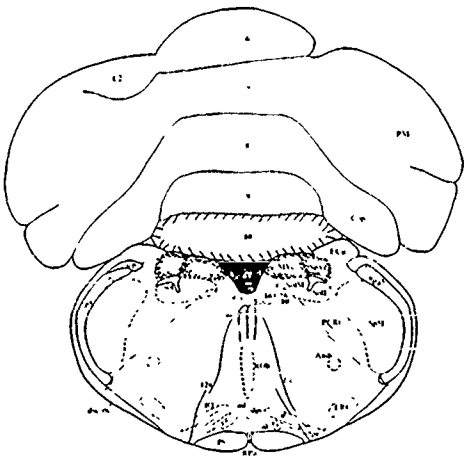
2 MIN

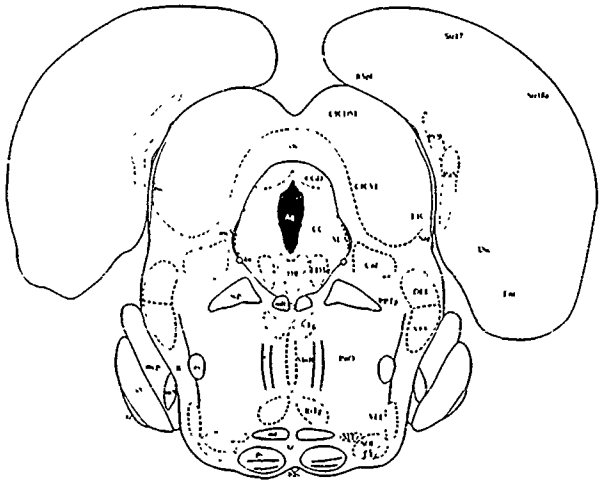
32 MIN



2 DAYS







4V fourth ventricle
~~10 dors motor nu of vagus~~
12 hypoglossal nu
12n root of hypoglossal nerve
Amb ambiguus nu
C2 crus 2 ansiform lobule
Cop copula pyramis
dsc dors spinocerebellar tr
ECu ext cuneate nu
Gi gigantocellular reticular nu
icp inf cerebellar peduncle
In interpositus cerebellar nu
IO inf olive
d = dors nu
dm = dorsomedial cell group
m = med nu
pr = principal nu
LRt lat reticular nu
ml med lemniscus
mlf med longitudinal fasciculus
~~MVe med vestibular nu~~
oc olivocerebellar tr
Pa5 paratrigeminal nu
PCRt parvocellular reticular nu
PM paramedian lobule
py pyramidal tr
ROb raphe obscurus nu
RPa raphe pallidus nu
sol solitary tr
SolL nu solitary tr, lat
SolM nu solitary tr, med
sp5 spinal tr trigeminal nerve
Sp5I nu spinal tr trigeminal nerve, interpositus
~~SpVe spinal vestibular nu~~
The ten lobules of the cerebellum are indicated by the numerals 1-10

4n trochlear nerve
Aq cerebral aqueduct
bas basilar artery
bic brachium inf colliculus
CG central grey
CGD central grey, dors
cic commissure inf colliculus
CICDM central nu inf colliculus, dorsomedial
CICVL central nu inf colliculus, ventrolateral
Cnf cuneiform nu
DLL dors nu lat lemniscus
DR dors raphe nu
Dsc lamina dissecans entorhinal cortex
EIC ext nu inf colliculus
Ent entorhinal cortex
LDTg laterodorsal tegmental nu
ll lat lemniscus
LTz lat nu trapezoid body
m5 motor root trigeminal nerve
mcp middle cerebellar peduncle
Me5 nu mesencephalic tr trigeminal nerve
me5 mesencephalic tr trigeminal nerve
ml med lemniscus
mlf med longitudinal fasciculus

MnR median raphe nu
MTz med nu trapezoid body
PaS parasubiculum
PnO pontine reticular nu, oral
PPTg pedunculopontine tegmental nu
PrS presubiculum
py pyramidal tr
rs rubrospinal tr
RSpl retrosplenial cortex
RtTg reticulotegmental nu pons
s5 sensory root trigeminal nerve
Sag sagulum nu
scp sup cerebellar peduncle
SOl sup olive
Str17 striate cortex, area 17
Str18a striate cortex, area 18a
ts tectospinal tr
tz trapezoid body
VLL vent nu lat lemniscus
VTg vent tegmental nu

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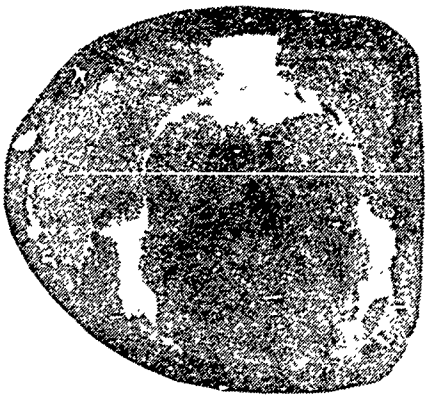
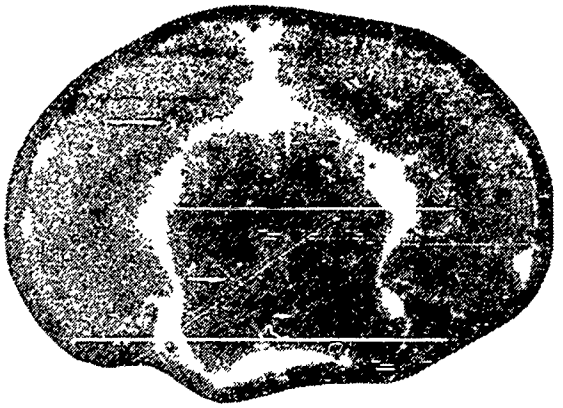
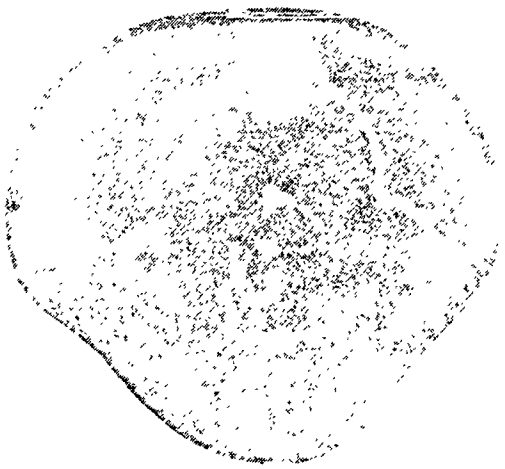
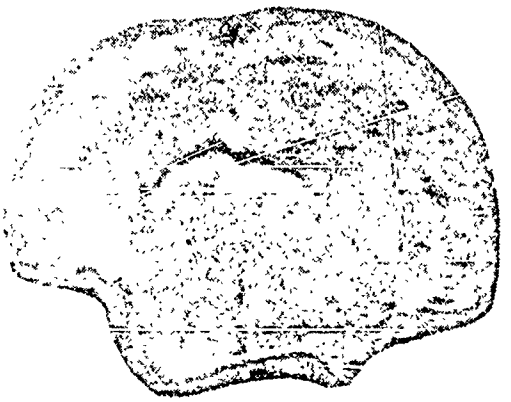
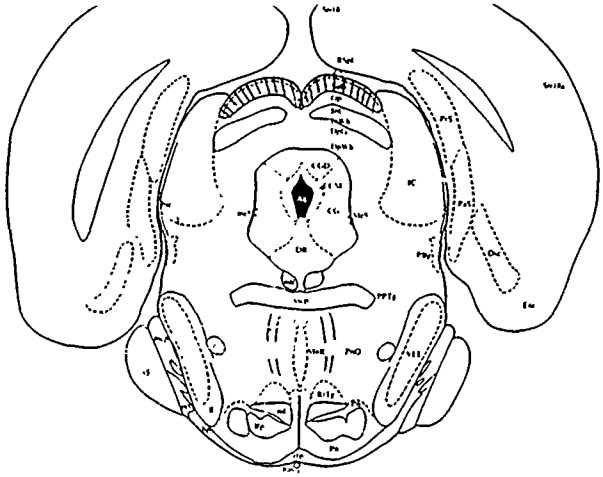


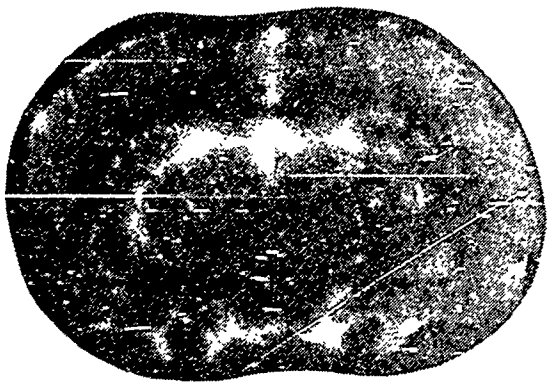


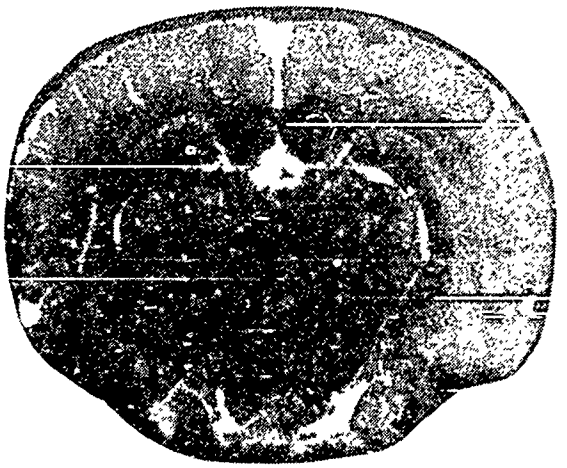


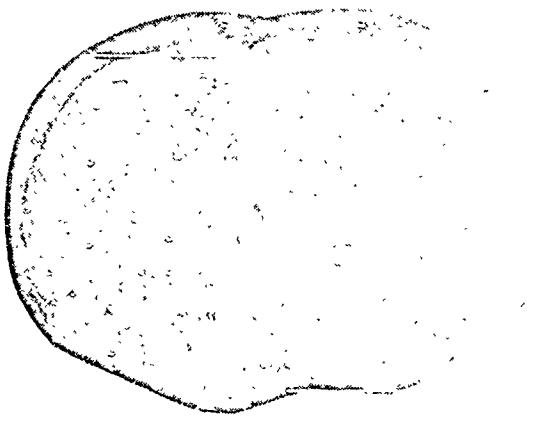


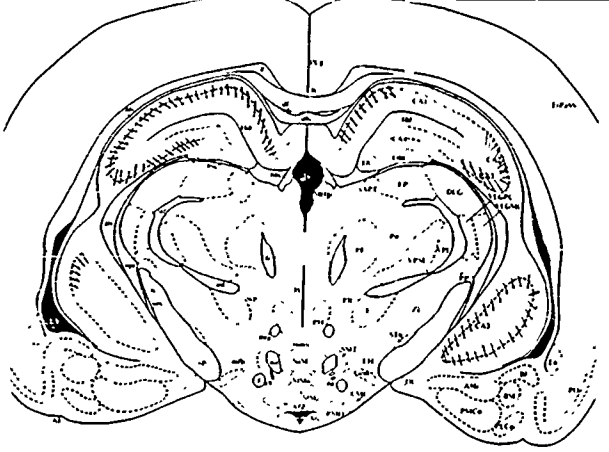


Aq cerebral aqueduct
bas basilar artery
bic brachium inf colliculus
bp brachium pontis
CG central grey
CGD central grey, dors
CGM central grey, med
DpG deep grey layer sup colliculus
DpWh deep white layer sup colliculus
DR dors raphe nu
Dsc lamina dissecans entorhinal cortex
Ent entorhinal cortex
IC inf colliculus
InG intermediate grey layer sup colliculus
InWh intermediate white layer sup colliculus
lfp longitudinal fasciculus pons
ll lat lemniscus
m5 motor root trigeminal nerve
mcp middle cerebellar peduncle
Me5 nu mesencephalic tr trigeminal nerve
me5 mesencephalic tr trigeminal nerve
ml med lemniscus
mlf med longitudinal fasciculus
MnR median raphe nu
Op optic nerve layer sup colliculus
PaS parasubiculum
PBg parabigeminal nu
Pn pontine nuclei
PnO pontine reticular nu, oral
PPTg pedunculopontine tegmental nu
PrS presubiculum
rs rubrospinal tr
RSpl retrosplenial cortex
RtTg reticulotegmental n pons
s5 sensory root trigeminal nerve
Str17 striate cortex, area 17
Str18 striate cortex, area 18
Str18a striate cortex, area 18a
SuG superficial grey layer sup colliculus
tfp transverse fibers pons
ts tectospinal tr
VLL vent nu lat lemniscus
xscp decussation sup cerebellar peduncle
Zo zonal layer sup colliculus









3V third ventricle
AF amygdaloid fissure
AHi amygdalohippocampal area
aiv aiveus hippocampus
APT ant pretectal area
Arc arcuate hy nu
BL basolateral amygdaloid nu
BM basomedial amygdaloid nu
bsc brachium sup colliculus
CA1 field CA1 of Ammon's horn
CA2 field CA2 of Ammon's horn
CA3 field CA3 of Ammon's horn
CA4 field CA4 of Ammon's horn
cc corpus callosum
cg cingulum

CMC caudal magnocellular nu hypothalamus
cp cerebral peduncle, basal
df dors fornix

DG dentate gyrus
dhc dors hip commissure
DLG dors lat geniculate nu
ec ext capsule
En endopiriform nu
F nu fields of Forel
f fornix
fr fasciculus retroflexus
FrPaSS frontoparietal cortex, somatosensory area
Gem gemini nuclei

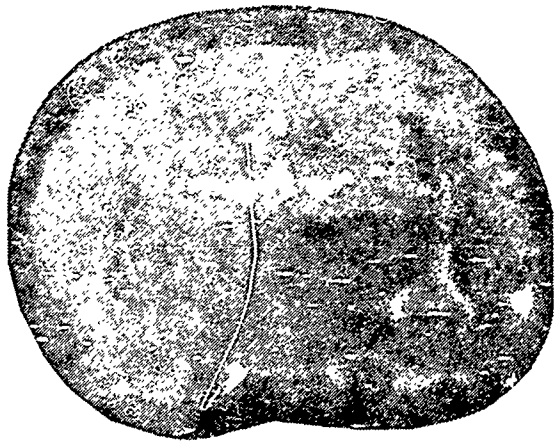
hbc habenular commissure
HiF hip fissure
IBl inner blade dentate gyrus
ic int capsule
IG induseum griseum
LH lat hy area
LP lat post th nu
LV lat ventricle

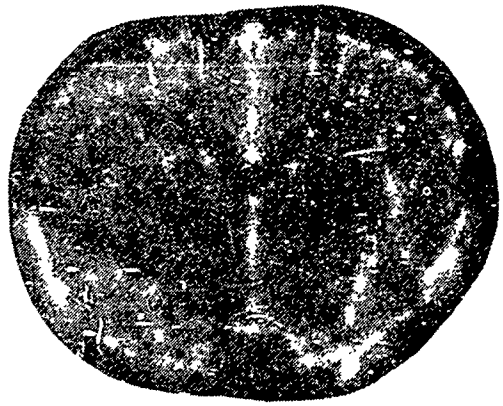
mfb med forebrain bundle
MHb med habenular nu
ml med lemniscus
MM med mammillary nu, med
MMn med mammillary nu median
MRe mammillary recess third ventricle

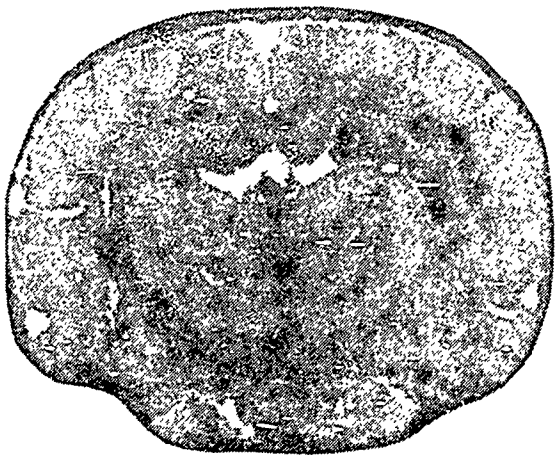
mt mammillothalamic tr
mtg mammillotegmental tr
OBl outer blade dentate gyrus
opt optic tr
PCg post cingulate cortex
PF parafascicular th nu
PH post hy nu
PLCo posterolateral cortical amygdaloid nu
PMCo posteromedial cortical amygdaloid nu
PMD premammillary nu, dors
PO primary olf cortex
Po post th nuclear group
PP peripeduncular nu

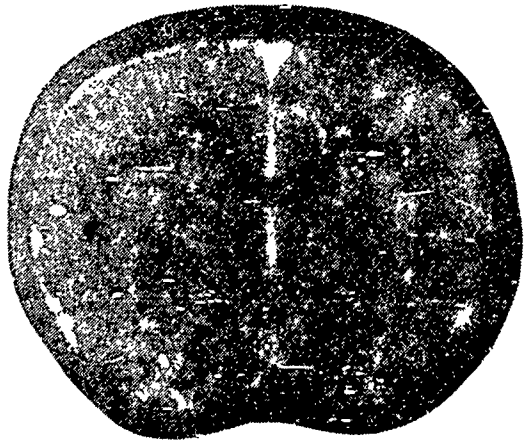
PR prerubral field
RF rhinal fissure
scp sup cerebellar peduncle
SMT submammillothalamic nu
sox supraoptic decussation
STh subthalamic nu
str sup th radiation
SuM supramammillary nu
sumx supramammillary decussation
VLGMC vent lat geniculate nu, magnocellular
VLGPC vent lat geniculate nu, parvocellular
VPL ventroposterior th nu, lat
VPM ventroposterior th nu, med

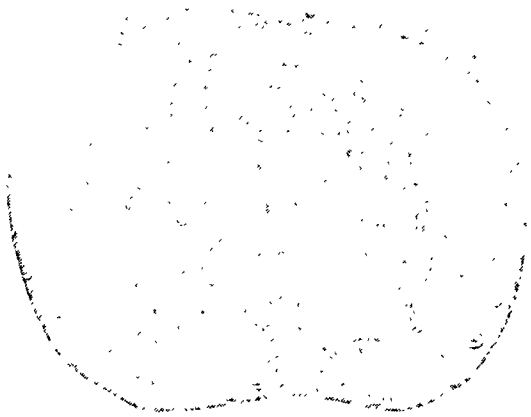
ZI zona incerta

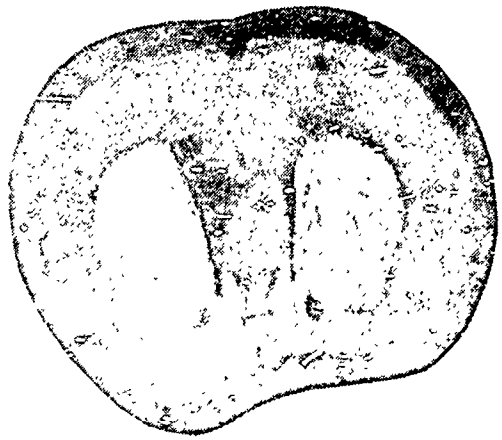


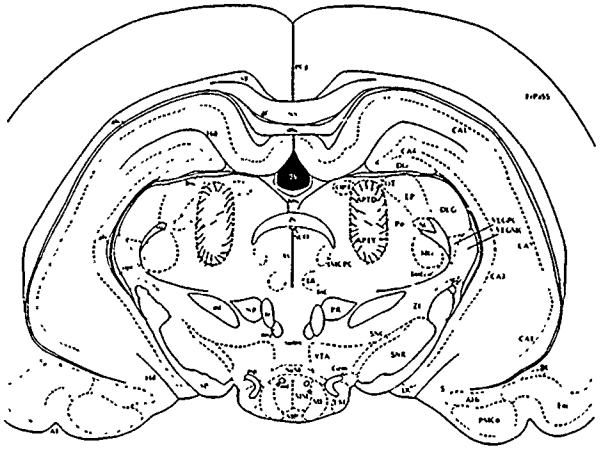


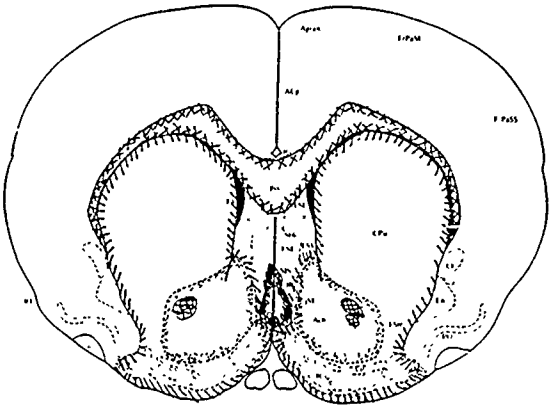












3V third ventricle
AF amygdaloid fissure
AHi amygdalohippocampal area
alv alveus hippocampus
~~AP(D) ant pretectal area, dors~~
~~AP(V) ant pretectal area, vent~~
BL basolateral amygdaloid nu
bsc brachium sup colliculus
CA1 field CA1 of Ammon's horn
CA2 field CA2 of Ammon's horn
CA3 field CA3 of Ammon's horn
CA4 field CA4 of Ammon's horn
cg cingulum
cp cerebral peduncle, basal
df dors fornix
DG dentate gyrus
dhc dors hip commissure
Dk nu of Darkschewitsch
DLG dors lat geniculate nu
Ent entorhinal cortex
fr fasciculus retroflexus
FrPaSS frontoparietal cortex, somatosensory area
Gem gemini nuclei
hbc habenular commissure
HiF hip fissure
IMCPC interstitial magnocellular nu post commissure
InC interstitial nu of Cajal
IntG intermediate geniculate nu

2n optic nerve
~~Ent ant entorhinal cortex~~
Acb accumbens nu
ACg ant cingulate cortex
Agran agranular region frontoparietal cortex, motor area
cg cingulum
Cl claustrum
~~CPu caudate putamen~~
ec ext capsule
En endopiriform nu
FrPaM frontoparietal cortex, motor area
FrPaSS frontoparietal cortex, somatosensory area
FStr fundus striati
~~ICjM islands of Calleja, major island~~
ICj islands of Calleja
IG induseum griseum
lo lat olf tr
LSD lat septal nu, dors
LSI lat septal nu, intermediate
LSV lat septal nu, vent
LV lat ventricle
mfb med forebrain bundle
MS med septal nu
PO primary olf cortex
RF rhinal fissure
SHi septohippocampal nu
Tu olf tubercle
~~VDB diagonal band~~
VP vent pallidum

LM lat mammillary nu
LP lat post th nu
MG med geniculate nu
ML med mammillary nu, lat
ml med lemniscus
MM med mammillary nu, med
MP med mammillary nu, post
mp mammillary peduncle
mt mammillothalamic tr
mtg mammillotegmental tr
OPT olivary pretectal nu
opt optic tr
OT nu optic tr
pc post commissure
PCg post cingulate cortex
PMCo posteromedial cortical amygdaloid nu
Po post th nuclear group
PP perpeduncular nu
PR prerubral field
RF rhinal fissure
S subiculum
scc splenium corpus callosum
SCO subcommissural organ
scp sup cerebellar peduncle
SG supragenicular th nu
SNC substantia nigra, compact
SNR substantia nigra, reticular
str sup th radiation
SuM supramammillary nu

sumx supramammillary decussation
VLGMC vent lat geniculate nu, magnocellular
VLGPC vent lat geniculate nu, parvocellular
VTA vent tegmental area
ZI zona incerta

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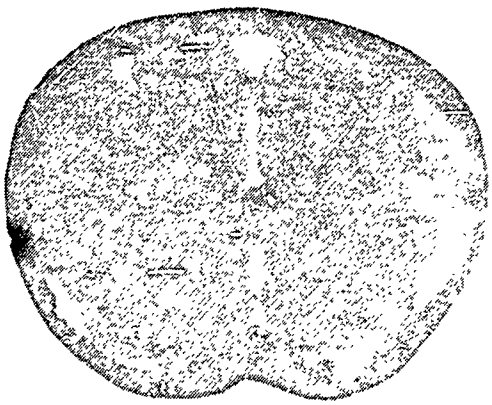
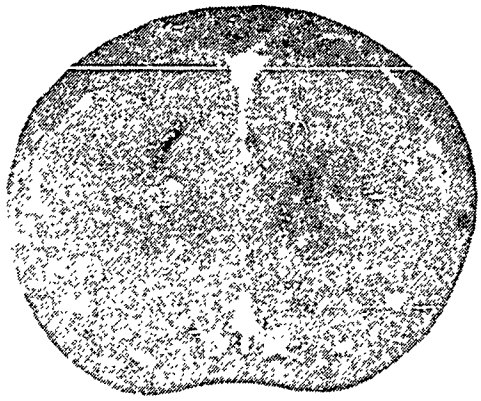
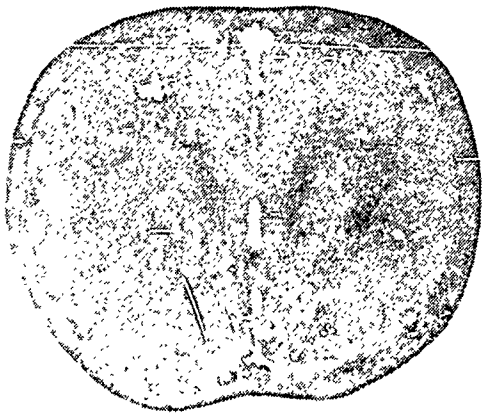
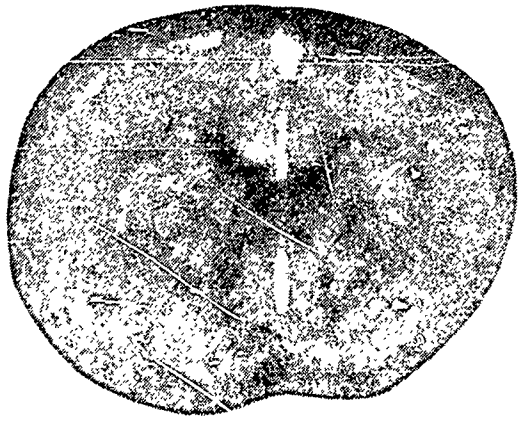
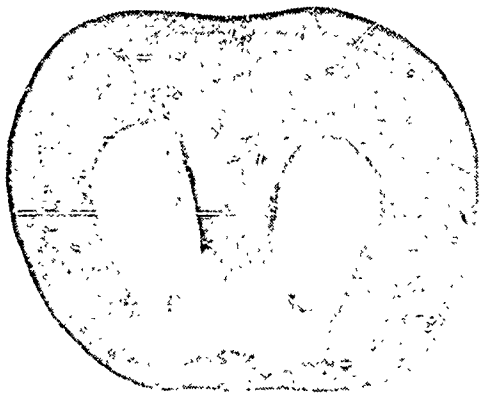
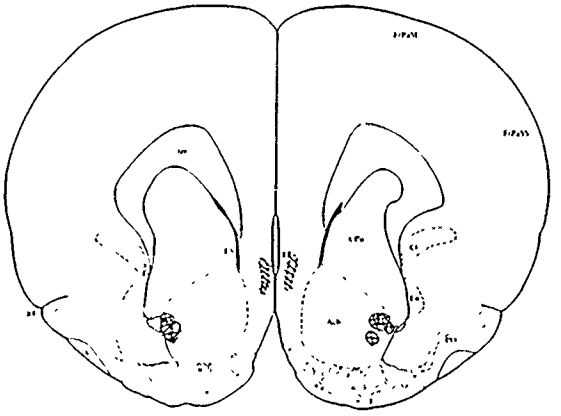




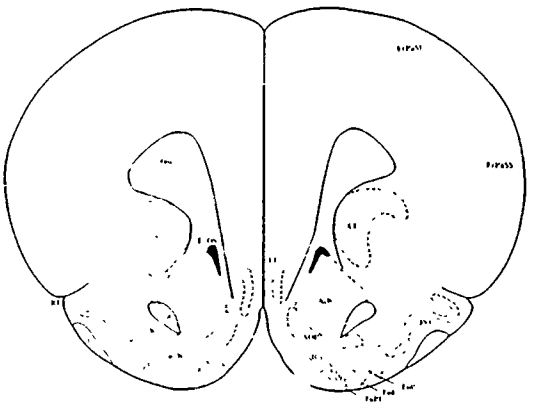








aca ant commissure, ant
Acb accumbens nu
Cl claustrum
CPu caudate putamen
En endopiriform nu
fmi forceps minor corpus callosum
FrPaM frontoparietal cortex, motor area
FrPaSS frontoparietal cortex, somatosensory area
ICj islands of Calleja
lo lat olf tr
LV lat ventricle
mfb med forebrain bundle
PO primary olf cortex
RF rhinal fissure
TT taenia tecta
Tu olf tubercle



aca ant commissure, ant
Acb accumbens nu
AOP ant olf nu, post
Cl claustrum
E ependyma and subependymal layer
fmi forceps minor corpus callosum
FrPaM frontoparietal cortex, motor area
FrPaSS frontoparietal cortex, somatosensory area
ICj islands of Calleja
lo lat olf tr
mfb med forebrain bundle
OV olf ventricle
PO primary olf cortex
RF rhinal fissure
TT taenia tecta
TuPl olf tubercle, plexiform layer
TuPo olf tubercle, polymorph layer
TuPy olf tubercle, pyramidal layer

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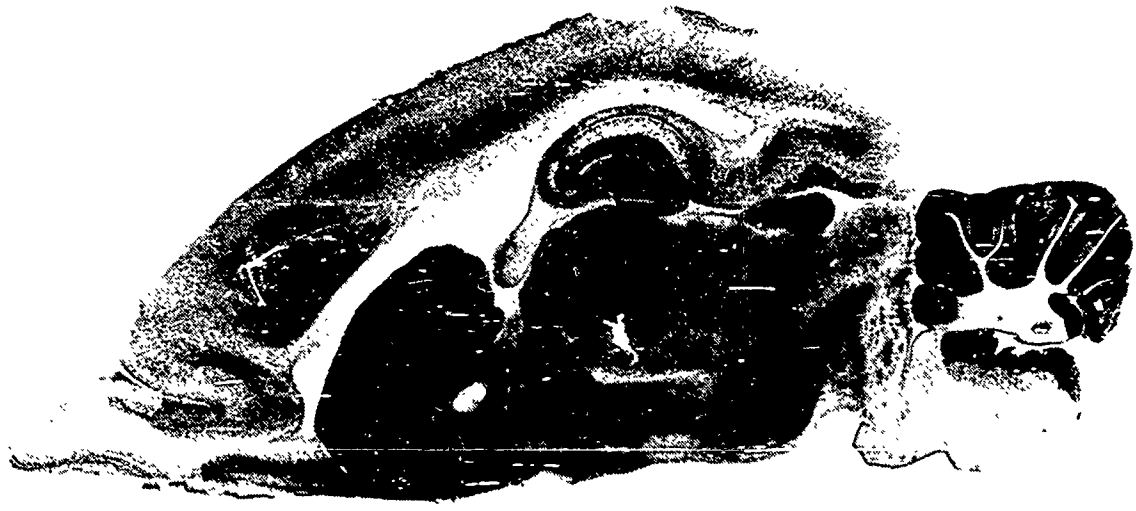
ACETYLCHOLINESTERASE DISTRIBUTION AND SOMAN BINDING IN THE BRAIN OF GUINEA PIG

R.E. Foster, K.R. Olson, R.R. Pindzola and M.T. Shipley
US Army Medical Research Institute of Chemical Defense
Aberdeen Proving Ground, Maryland 21010-5425

INTRODUCTION

The central nervous system effects of organophosphorous compounds such as pinacolyl methylfluorophosphonate (soman) are dependent on distribution to "sites of action". In order to better understand the distribution of soman after systemic exposure, we have sought to define the total pattern of covalent binding sites by *in vitro* labeling of serial brain sections. Tritiated-soman was utilized in order to produce highly accurate tissue autoradiograms which define the areal distribution (i.e. localization or total pattern) of covalent binding sites. Correlative acetylcholinesterase assays of tissue homogenates and tissue sections provided points of reference for interpreting the soman binding distribution. This total pattern of covalent soman binding to guinea pig brain sections will be compared to binding after systemic exposures in future experiments. Understanding any differences between the total binding distribution as defined in the present study and the distribution after systemic exposure will be a step in the direction of comprehending the functional effects of soman.

A



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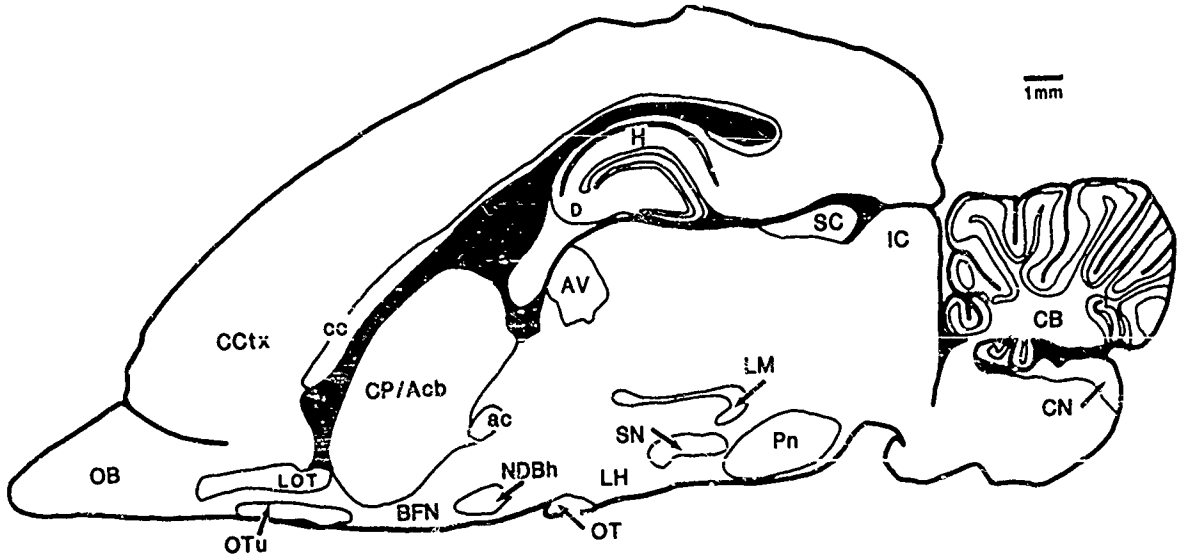
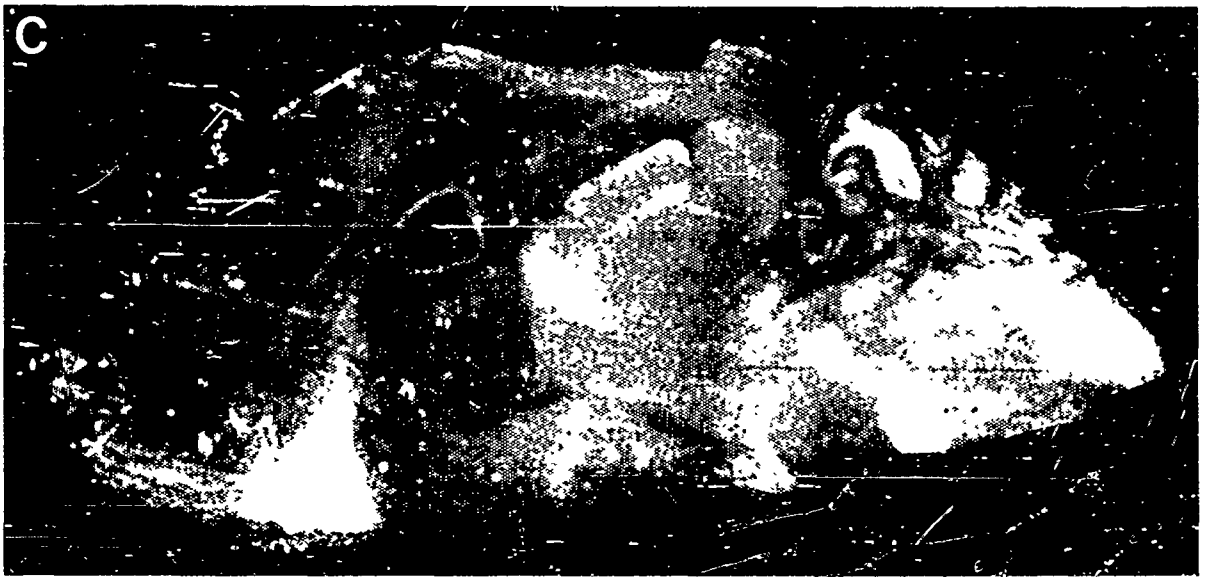
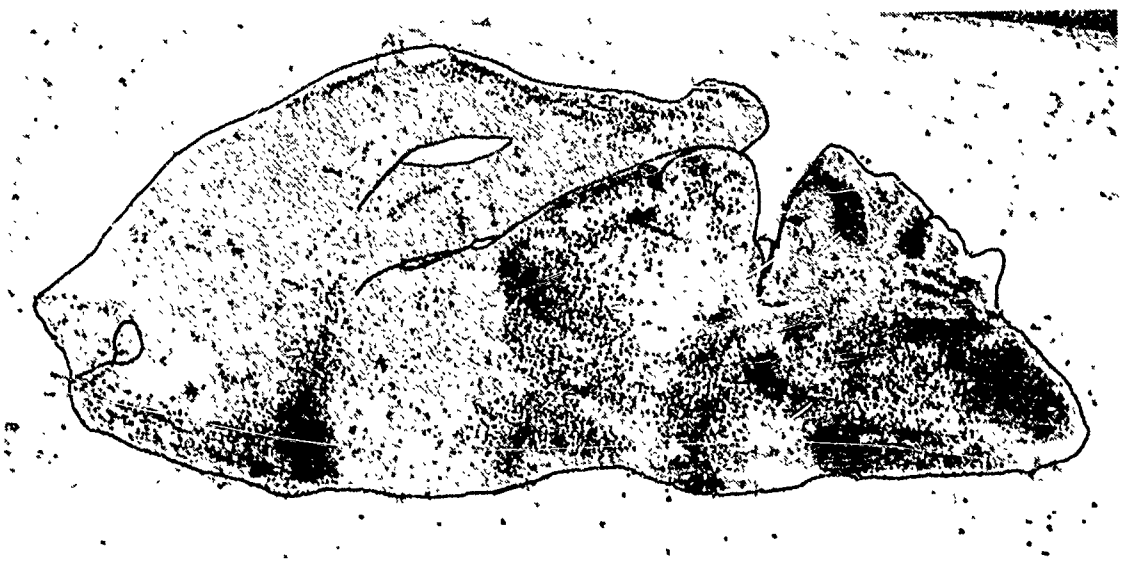

1mm
H
D
SC
IC
AV
CB
CCtx
cc
CP/Acb
LM
ac
SN
Pn
CN
OB
LOT
NDBh
LH
BFN
OT
OTu

C



D



AChE Distribution

BRAIN AREAS	RADIOMETRIC ASSAY (SIKKOTOS ET AL., 1969)[1]			COLORIMETRIC ASSAY (MODIFIED ELLMAN ET AL., 1961)[2]		
	μmoles/minute/G wet weight ± S.E.M.			μmoles/minute/G wet weight ± S.E.M.		
	MCh[3]	ACh[4,5]		Acetylthiocholine iodide[5,6]		
	FRESH[7] (n=5)	SALINE[8] (n=3)	FRESH[7] (n=5)	FRESH[7] (n=5)	SALINE[8] (n=5)	FIXED[9] (n=5)
Cerebral cortex	1.5±0.1	2.7±0.2	2.0±0.2	8.9±4.4	10.2±0.5	2.2±1.8
Hippocampus	7.7±1.1	18.5±4.3	10.1±8.4	7.8±1.9	11.1±0.5	1.0±0.6
Thalamus	9.8±1.0	21.5±1.5	14.3±1.4	11.3±1.9	14.6±0.4	0.5±0.3
Olfactory Bulb	13.8±2.1	21.6±3.8	18.0±2.1	2.9±1.3	4.3±0.4	3.5±3.1
Midbrain	12.7±1.5	20.3±4.0	18.2±1.8	14.3±2.4	17.9±0.5	1.0±0.7
Medulla	16.6±3.6	28.6±1.1	25.4±1.2	13.8±3.5	17.7±4.3	1.0±0.5
Cerebellum- vermis	27.0±2.7	44.9±5.4	41.2±5.3	22.0±8.9	24.7±1.3	2.2±1.8
Cerebellum- neocortex	29.9±4.1	30.9±4.1	45.8±3.5	22.4±8.9	20.3±0.5	1.3±0.9
Cervical spinal cord	43.4±4.9	29.9±1.5	66.0±4.8	16.3±4.4	16.5±0.6	0.9±0.6
Striatum	93.5±11.7	83.0±13.6	159.1±16.1	145.0±7.6	36.3±3.3	3.3±2.6
Whole Brain	4.2±0.12		6.0±0.15	14.1±1.2[10]		

1 Sikkotos, A N , Filbert, M , and R Hester Biochem Med 3 1-12, 1969

2 Ellman, G L , Courtney, K D Andres, V , and R M Featherstone Biochem Pharmacol 7 88-95, 1961 as modified by Brogdon W G and C ** Dickinson Anal Biochem 131 499-503 1983

3 Total volume= 300 μl, 20 μl sample, 180 μl 0 1M phosphate buffer, 100 μl ^{14}C-methacholine, 5 min incubation

4 Same as #3 except ^{14}C-acetylcholine was the substrate

5 Less than 5% of the activity was attributable to butyrylcholinesterase

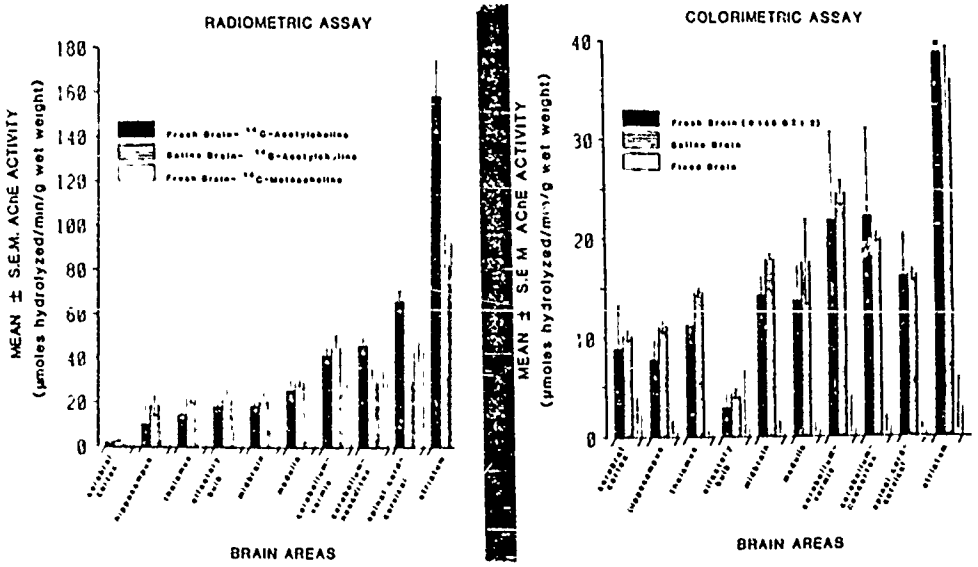
6 Total volume= 300 μl, 100 μl sample 200 μl substrate + color reagent (1mM DTNB in 0 1M phosphate buffer + 1mM acetylthiocholine iodide)

7 Fresh brains removed from skull on ice dissected into areas weighed, and frozen Thawed tissue was homogenized with pestle to a 10% solution in 1% Triton X-100 in water Homogenates were spun down and the supernatant

8 Saline brains were treated as in #7 except that the blood was removed from all tissues by transcardiac perfusion with physiological saline solution

9 Fixed brains were treated as in #7 except that the brain was exposed to 30 minutes of mixed aldehyde fixation initiated by transcardiac perfusion of saline followed by the aldehyde fixative (1% paraformaldehyde - 1 25% gluteraldehyde in 0 1M phosphate buffer)

10 Samples from these brains were independently assayed by Analytical Chemistry Branch using an automated Ellman assay procedure The activity value obtained = 9 9 μmoles AcThio hydrolyzed/min/G wet weight @ 37°C



AChE and Soman Binding Localization

COMPARABLE SAGITTAL SECTIONS THROUGH THE GUINEA PIG BRAIN ARE SHOWN AFTER DIFFERENT TREATMENTS.

A. Modified Koelle stain showing the distribution of acetylcholinesterase.

B. Line drawing of a Nissl stained section for orientation and cytoarchitectonic boundaries.

C. Darkfield image of parasagittal section labeled with tritiated-soman (emulsion-Kodak NTB-3, 7 week exposure).

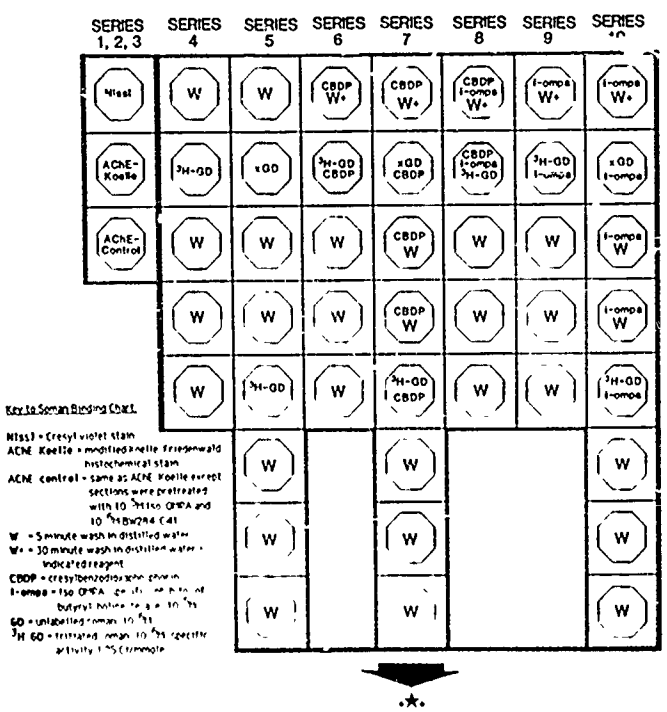
D. Color enhanced computer reconstruction of a digitized autoradiogram (illustrated in C) illustrating the relative density of covalent soman binding.

LIST OF ABBREVIATIONS

ac	Anterior commissure
Acb	Nucleus accumbens
AV	Anteroventral nucleus of the thalamus
BFN	Basal forebrain nuclei
CB	Cerebellum
cc	Corpus callosum
CCtx	Cerebral cortex
CN	Cochlear nucleus
CP	Caudate-putamen
D	Dentate gyrus
Dth	Dorsal thalamus
F	Fornix
H	Hippocampus
hc	Hippocampal commissure
IC	Inferior colliculus
ICc	Inferior colliculus, central nucleus
LH	Lateral hypothalamus
LM	Medial lemniscus
LOT	Lateral olfactory tract
MS	Medial septal nucleus
NDBh	Nucleus of the diagonal band, horizontal limb
NDBv	Nucleus of the diagonal band, vertical limb
OB	Olfactory bulb
OT	Optic tract
OTu	Olfactory tubercle
PBA	Parabrachial area
Pn	Pontine nuclei
PRT	Pretectal area
SAI	Stratum album intermediale
SAP	Stratum album profundum
SC	Superior colliculus
SGI	Stratum griseum intermediale
SGP	Stratum griseum profundum
SGS	Stratum griseum superficiale
SN	Substantia nigra
SO	Stratum opticum
V	Motor nucleus of the trigeminal nerve
VII	Nucleus of the facial nerve

Soman Binding Procedure

1. Serial 20 µm cryostat sections of fresh frozen guinea pig brain were separated into 10 series (i.e., every 10th section = 1 series
2. Section series were processed processed in one of three ways: a) Nissl stain for visualization of cytoarchitecture:
   b) modified Koelle stain for visualization of acetylcholinesterase
   c) incubated in ^{3}H-soman for determination of binding sites.
3. Protocols for ^{3}H-soman incubation, autoradiography & scintillation counting.



DEHYDRATION-DEFATTING

Air Dryed Sections

40% ETOH --- 5 min.
70% ETOH --- 5 min.
80% ETOH --- 5min
95% ETOH --- 5 min.
100% ETOH --- 5 min
100% ETOH --- 5 min
Xylene ------- 5 min
Xylene ------- 1 hr.
Xylene ------- 5 min.
100% ETOH --- 5 min
100% ETOH --- 5 min
95% ETOH --- 5 min
80% ETOH --- 5 min
70% ETOH --- 5 min
40% ETOH --- 5 min
Dist H_2O --- 5 min.
Air Dry ----- Until Used

AUTORADIOGRAPHY*

1. Coat section with Kodak NTB-3 nuclear tract emulsion
2. Expose in dark for 6 weeks.
3. Develop, counterstain, and coverslip

SCINTILLATION COUNTING*

1. Scrape section from slide.
2. Weigh
3. Suspend in ULTRAFLUOR.
4. Count for 10 minutes.

*Every other set of sections in series 4 - 10 (i.e., sections #4 - #10 for scintillation, sections #14 - #20 for autoradiography)

SOMAN BINDING SITES

1. Calculation of soman binding for 'series 4' sections (total binding) via scintillation assay.

A. Assumptions:
   a) Single parasagittal sections have AChE levels representative of whole brain homogenate levels;
   b) Activity of rat brain AChE is similar to that from guinea pig brain.
   c) One mole of soman binds to one mole of AChE.

B. Given or Measured:
   a) Weight of tissue from a 20 um section, mean = 129.8 ug/section (n=14);
   b.) Specific activity of the labelled soman, 1.25 Ci/mmole which yields 2.75 DPM/fmole of soman.
   c.) Mean DPM/section = 124;
   d.) AChE activity in whole brain homogenate 0.6 um ACh hydrolyzed/mg wet weight/min.
   e.) Activity of 1 mg of rat brain AChE (@ pH 7.3, 25°C), 4000 um ACh hydrolyzed/min.

C. How much AChE/section? 1.8×10^{-6} mg AChE/section =

$$\left(\frac{0.6 \text{ um ACh/mg tissue}}{4000 \text{ um ACh/mg AChE}} \right) \times 12 \text{ mg/section}$$

D. What is [AChE]/section? 22.5 fmoles AChE/section =

$$\frac{1.8 \times 10^{-6} \text{ mg AChE/section}}{9.0 \times 10^{4} \text{ mg AChE/mmole}} = 2.25 \times 10^{-11} \text{ mmoles/section}$$

E. At 124 DPM/section, what percentage of the label is attributable to AChE binding?

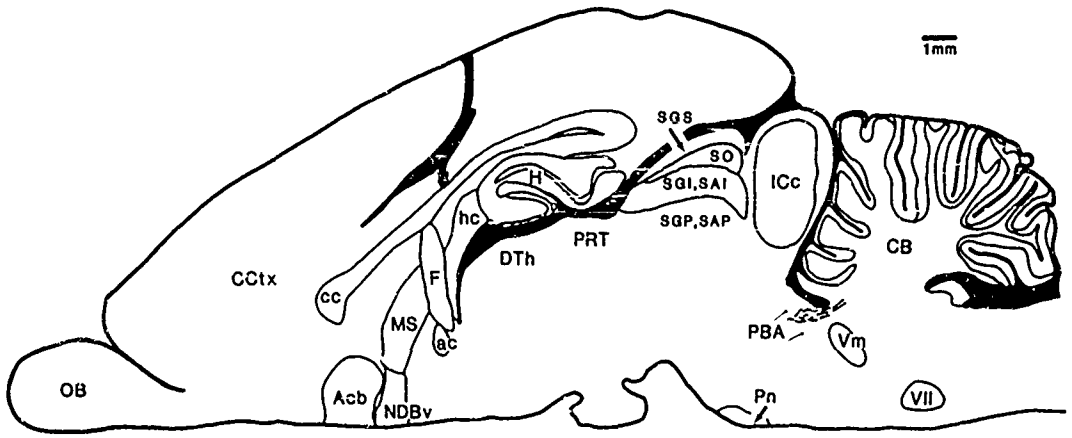
$$\sim 50\% = \frac{124 \text{ DPM/section}}{22.5 \text{ fmole AChE/section} \times 2.75 \text{ DPM/fmole soman}}$$

2. DPM/section for 'series 6 & 8' ≈ 60.

3. THUS, by biochemical blocking of non-specific esterases (2) and by calculation (1E), there is an approx. 1:1 ratio of 'non-specific' to 'specific (AChE)' covalent binding of soman in guinea pig brain.

A

B


1mm
SGS
SO
SGI,SAI
SGP,SAP
H
hc
PRT
DTh
ICc
CB
CCtx
cc
F
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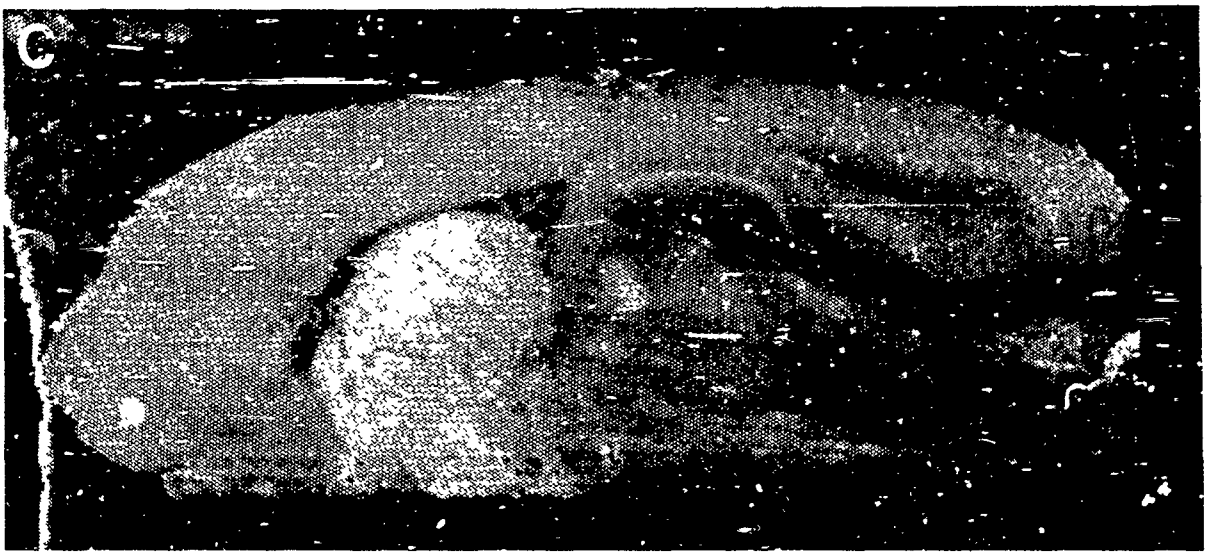
ac
PBA
Vm
OB
Acb
NDBv
Pn
VII



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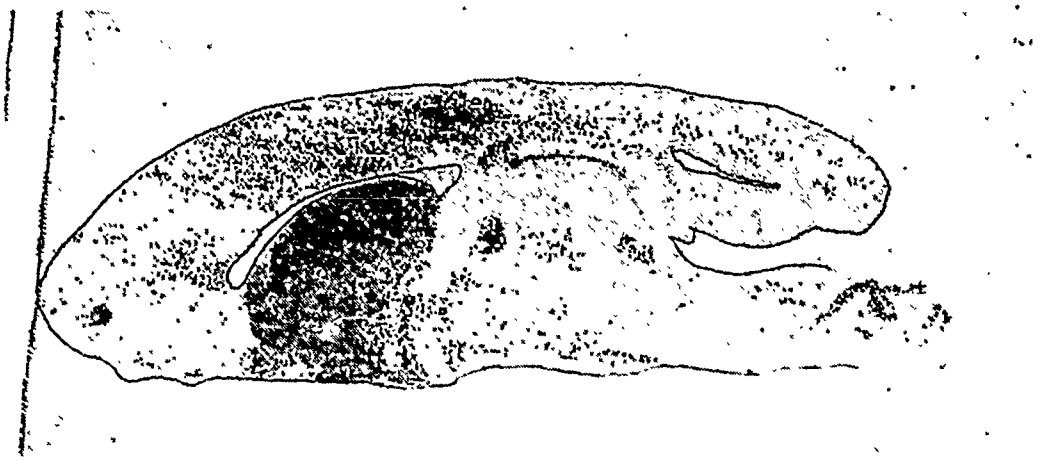

CONCLUSIONS

1. BINDING. Approximately one-half of the covalent binding of soman to guinea pig brain is attributable to acetylcholinesterase sites and the remaining is attributable to "non-specific" or CBDP-sensitive sites.

2. LOCALIZATION. From tissue autoradiograms, there appears to be a uniform areal distribution of covalent binding sites for soman throughout the brain with the exception of greater label distribution over those brain areas which have high levels of acetylcholinesterase activity (e.g., caudate nucleus, nucleus accumbens, cranial nerve nuclei, etc).

CORRELATION OF 2-PAM PLASMA LEVELS AFTER ORGANOPHOSPHATE INTOXICATION

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INTRODUCTION

Pralidoxime, or 2-PAM, is an antidote which has found wide acceptance in the treatment of acute organophosphorus poisoning. Pralidoxime is effective in reactivating non-aged phosphorylated cholinesterase enzyme and thereby restoring function to an otherwise inhibited enzyme. Pralidoxime is largely excreted by glomerular filtration and active transport in the kidney (1,2), and normally little of the compound is metabolized in vivo (3). Pralidoxime is not significantly bound to plasma proteins (4). Being a charged compound, 2-PAM slowly penetrates plasma membranes, RBCs (5) and the blood-brain barrier (4). Due to this latter characteristic, Fleisher (6) suggested that the therapeutic efficiency of an oxime primarily depends on its ability to act on cholinesterase molecules located on the surface of tissue membranes.

Pralidoxime has been a clinical and experimental entity for many years, and its plasma concentration versus time relationship has been observed in many studies (7-10). However, until recently the influence of organophosphorus inhibitors on plasma levels of 2-PAM has not been examined. The experiments described in this poster represent current, continuing efforts on the effects of potent, highly toxic inhibitors of cholinesterase on the plasma concentration of antidote compounds. In fact, since potent inhibitors of cholinesterase, such as soman and sarin, profoundly alter the physiology of animals, one should expect changes in the absorption, distribution, metabolism, and elimination of therapeutic drugs, following organophosphorus poisoning.

METHODS

Guinea pigs were injected with either sarin (GB) or soman (GD) subcutaneously and one minute later were given 2-PAM chloride and atropine sulfate intramuscularly in the same hind limb. Animals were injected with either 6.25 or 25 mg/kg of 2-PAM and 16 mg/kg of atropine. Different groups of guinea pigs were injected with a selected level of organophosphorus compound based on multiples of their lethal dose 50 (xLD50) value. These multiples were 0.7, 1.2, 2.2, 2.8, 5.6 and 11.2. Following the injection of the therapeutic compounds, animals were decapitated at 1, 2, 4, 6 and 10 minutes. Trunk blood was analyzed for 2-PAM content by an automated spectrophotometric technique based on the method of Groff and Ellin (10). Both plasma concentrations for 2-PAM from individual as well as groups of animals were used in the analysis of the data. Each mean represents the average of 5-7 animals.

Due to the dispersion in the data, resulting from a destructive sampling technique, a rigorous pharmacokinetic analysis of the data was not attempted. Rather, the data were analyzed for non-compartmental measures, such as time to peak, maximal plasma concentration (CP_{max}) and area under the curve (AUC).

Plasma concentrations of 2-PAM were analyzed for statistical significance using a 2-way ANOVA method. Time and xLD50 level of challenge were used as factors in the statistical analysis. Area under the curve was computed from the mean of the plasma concentrations by the trapezoid rule between one to ten min. The CP_{max} was defined as the highest mean plasma concentration observed over the experimental period. Control levels were determined for each dosage of 2-PAM in three separate trials and pooled. To maximize the information describing the relationship between the various challenge levels of GB and GD, both CP_{max} and AUCs were normalized as a percentage of control trails.

RESULTS

In animals poisoned with either GB or GD and following the injection of 2-PAM and atropine, plasma levels of 2-PAM increased and decreased in similar time course regardless of the xLD50, dosage of 2-PAM or oganophosphorus poison. However, both the challenge level of GB or GD and dosage of 2-PAM strongly influenced the magnitude of the CP_{max} and AUC. Similar effects of GD on plasma levels of 2-PAM have been reported earlier (11). Generally, levels of xLD50 equal to or greater than 2.8, tended to increase both CP_{max} and AUC. In contrast, levels of the xLD50 equal to or lower than 2.2 tended to decrease both CP_{max} and AUC. Representative results of the effects of xLD50s for GB and GD on plasma concentrations of 2-PAM are presented in Fig. 1 and 2.

The CP_{max} was observed at 2 min in 80% of all trials. In the remaining 20% of the trials, the CP_{max} was observed at 4 min. The time to CP_{max} was not associated with either the dosage of 2-PAM or xLD50 challenge in a systematic fashion.

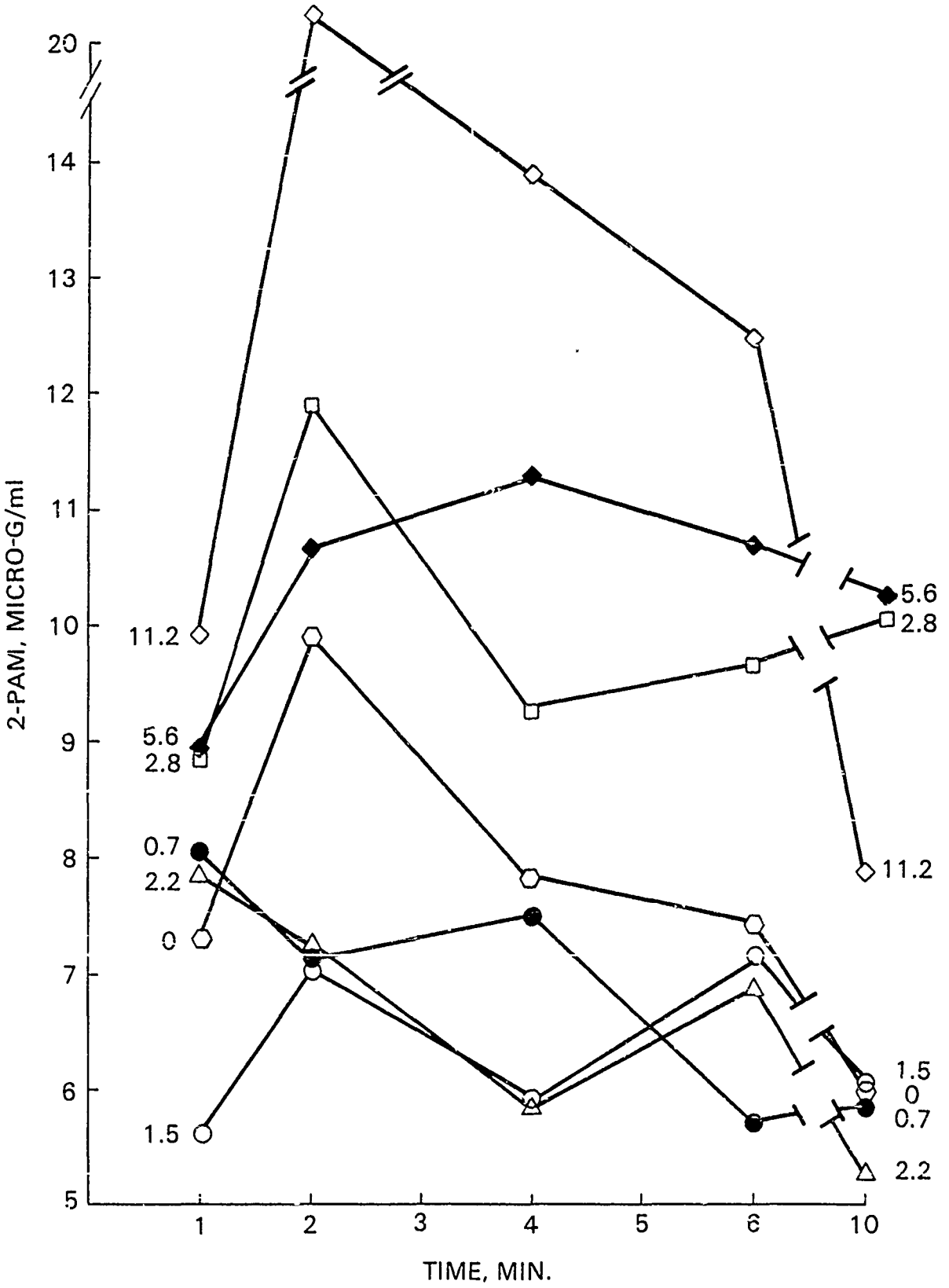
Using a 2-way ANOVA technique, the individual plasma levels of 2-PAM were analyzed. A significant relationship was found for time and xLD50 levels for both 6.25 and 25.0 mg/kg of 2-PAM with $p < 0.001$ and $p < 0.00001$, respectively.

Since GB and GD had similar effects on the plasma concentrations of 2-PAM for both 6.25 and 25.0 mg/kg dosages, the data from all sets of experiments were normalized as a percentage of control trials. The normalized data for CP_{max} and AUC were combined for all sets of data. Thus an average CP_{max} (CP_{max}) and an average AUC (AUC) were computed encompassing the CP_{max} of the four different experimental conditions at each xLD50 tested. Over the entire range of xLD50 the maximum plasma concentrations (CP_{max}) were changed in value from 81 to 170%, as compared to control, and the AUCs were changed in value from 69 to 203%, as compared to control. The normalized data, its mean and standard deviation are presented in Table 1.

Using the data in Table 1, the effect of different xLD50 for GB and GD on AUC and CP_{max} was further characterized by classifying the changes in AUC and CP_{max} as an increase or decrease, as compared to control values. Next, the frequency with which any given xLD50 increased or decreased these values was calculated. Finally, increases or decreases were grouped and plotted as frequency of effect versus the xLD50 (see Fig. 3). An inspection of Fig. 3 reveals that the predominant effect of GB and GD below an xLD50 of 2.2 is one of decreasing both CP_{max} and AUC, whereas above an xLD50 of 2.8 is one of increasing both CP_{max} and AUC. In fact, at 0.7 xLD50, both CP_{max} and AUC were always decreased and at 5.6 and 11.2 xLD50 both CP_{max} and AUC were always increased relative to control values. The biphasic relationship of xLD50 to AUC or CP_{max} was further characterized by performing a linear regression of % AUC or % CP_{max} of control against xLD50 values, using the data from Table 1. Both % AUC and % CP_{max} were highly correlated with xLD50 with $r = 0.9845$ and $r = 0.9498$, respectively. These regressions are presented in Fig. 4 and 5.

FIGURE 1

PLASMA LEVELS OF 2-PAM FOLLOWING A DOSE OF 6.25 mg/kg AND VARIOUS xLD50 OF GD



AVERAGE COEFFICIENT OF VARIATION IS 10.5

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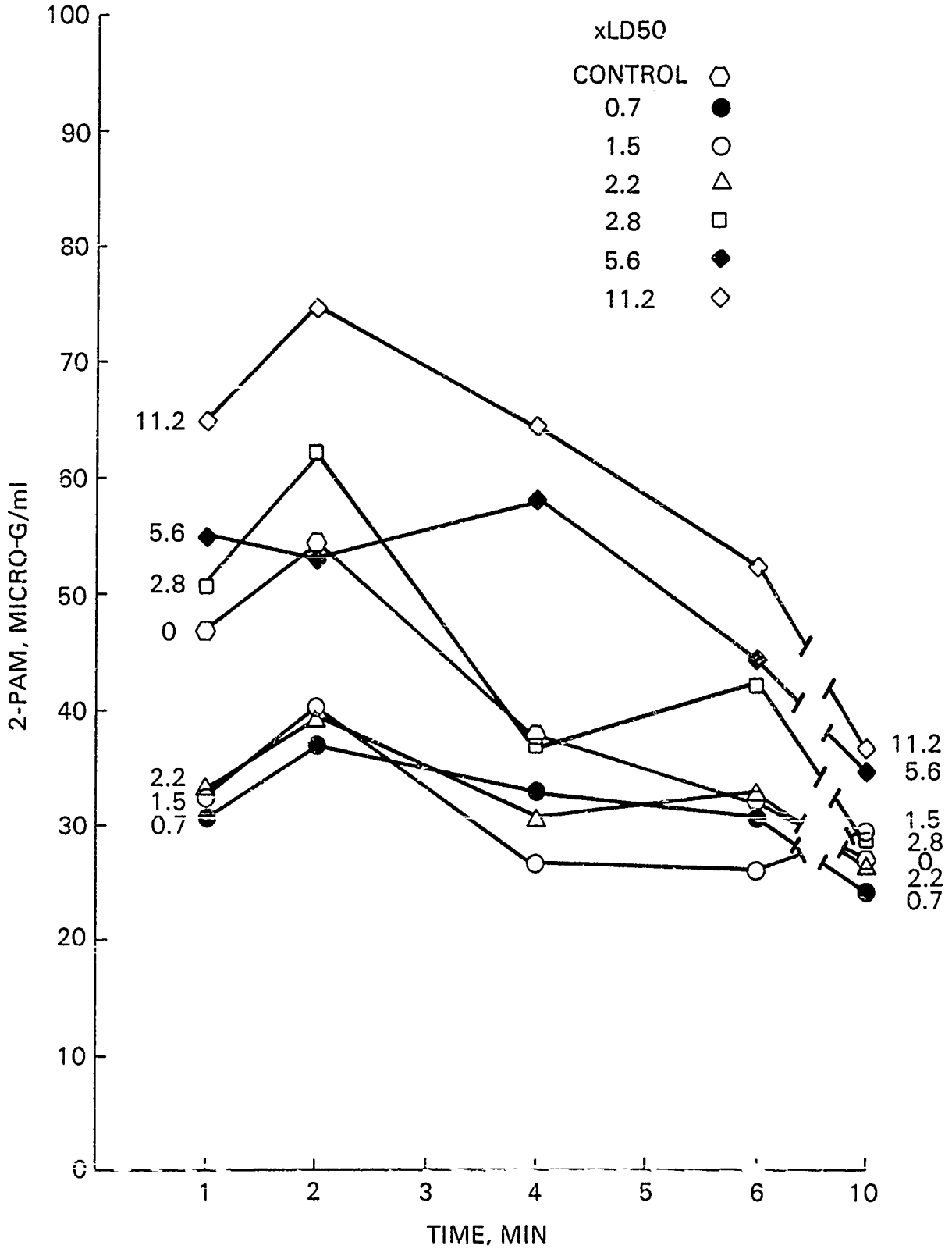
FIGURE 2

PLASMA LEVELS OF 2-PAM FOLLOWING A DOSE OF 25 mg/kg AND VARIOUS xLD50 OF GD



AVERAGE COEFFICIENT OF VARIATION IS 10.5.

A-735

TABLE 1

PERCENT CHANGE IN AUC

xLD50	25 mg/kg		6.25 mg/kg		
	GD	GB	GD	GB	$\bar{X} \pm SD$
0.7	83	87	86	99	89 ± 6
1.5	81	102	87	108	94 ± 12
2.2	87	116	84	126	103 ± 20
2.8	114	130	131	98	118 ± 15
5.6	128	146	140	120	133 ± 11
11.2	152	159	170	128	152 ± 17

CHANGES IN AUC AFTER GD OR GB AS A PERCENTAGE OF CONTROL AT 6.25 OR 25 mg/kg, 2-PAM.

TABLE 2

PERCENT CHANGE IN CP_{max}

xLD50	25 mg/kg		6.25 mg/kg		
	GD	GB	GD	GB	$\bar{X} \pm SD$
0.7	69	77	81	96	81 ± 11
1.5	75	80	72	106	83 ± 15
2.2	73	104	79	119	94 ± 21
2.8	114	125	119	97	114 ± 12
5.6	107	162	113	135	129 ± 24
11.2	137	185	203	182	177 ± 28

CHANGES IN CP_{max} AFTER GD OR GB AS A PERCENTAGE OF CONTROL AT 6.25 OR 25 mg/kg 2-PAM.

FIGURE 3

FREQUENCY OF EFFECT ABOVE AND BELOW CONTROL FOR CP_{max} OR AUC BASED ON MEAN RESPONSE

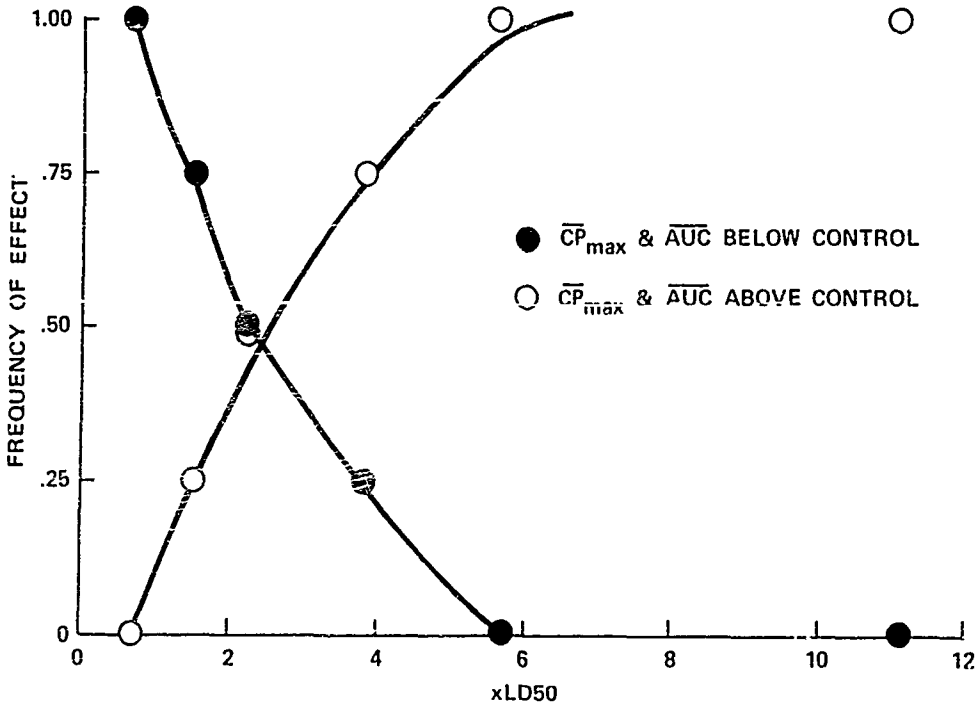


FIGURE 4

CORRELATION OF PERCENTAGE MEAN AUC AND xLD50

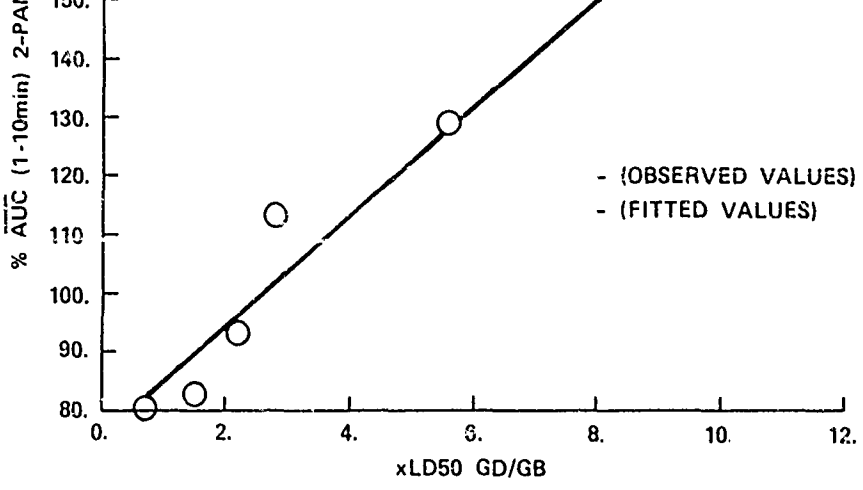


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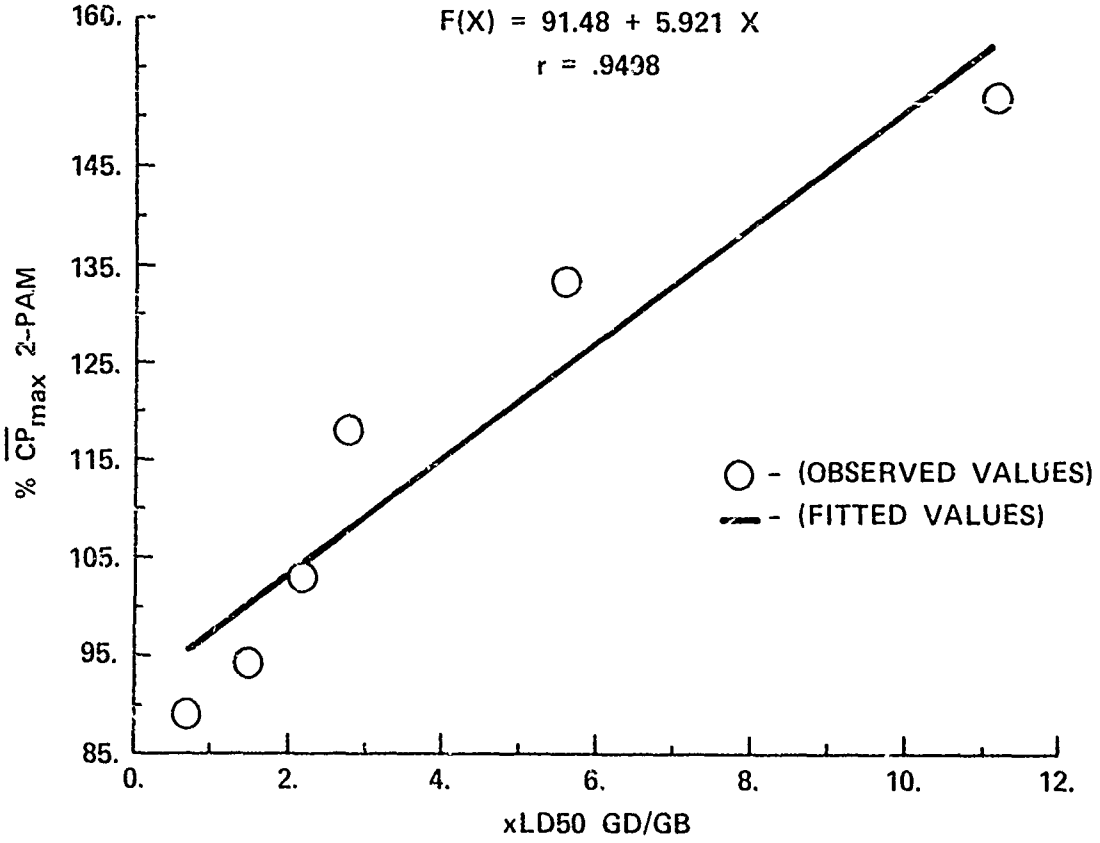
FIGURE 5

CORRELATION OF PERCENTAGE MEAN CP_{max} AND xLD50



SUMMARY

In summary, this work continues earlier work on the effects of GD and GB on the plasma levels of 2-PAM. This work extends earlier findings by quantifying the effects of nerve agents on plasma levels of 2-PAM over a wide range of toxicity. The different levels of toxicity were produced by using a range of challenge levels of nerve agents extending from 0.7 to 11.2 LD50 values. The findings of this study indicate that there is a systematic change in the influence of nerve agent challenge on the plasma level of 2-PAM. The systematic change is composed of a biphasic effect which, at low levels of challenge, decreases and, at high levels of challenge, increases plasma levels of 2-PAM. From a greater understanding of these influences of nerve agent poisoning on therapeutic drug concentrations, better models of in vitro and in vivo efficacy will be possible.

DISCUSSION

Typically the plasma concentrations of therapeutic compounds are determined in physiologically normal animals and the influences of a pathophysiological state on plasma levels are not considered. It is apparent from the work just described and from that reported earlier (11) that the pathophysiological state induced by GB or GD strongly influences the plasma concentration of 2-PAM. An understanding of the changes in plasma 2-PAM levels due to poisoning may contribute to an understanding of the pathophysiology of nerve agent poisoning, but also to a better understanding of the relationship between in vitro and in vivo concentrations of an oxime and efficacy. A practical consequence of this work is that plasma levels based on dosages of 2-PAM or other oximes in non-poisoned animals would lead to an inaccurate estimate of the plasma levels of oximes in poisoned animals. This would be true since GB and GD modify the pharmacokinetics of 2-PAM. In fact, the pathophysiological effects of GB and GD appear to be biphasic and dependent on the challenge level of the nerve agent (see Fig. 1 and 2). When the challenge levels were quantified in terms of a multiple of the LD50 value, which ranged from 0.7 to 11.5, the effects of nerve agent on plasma levels of 2-PAM could be classified as either increasing or decreasing the CP_{max} or AUC at any xLD50 relative to control values (see Table 1) for any given dosage of 2-PAM. Thus all the data could be normalized and used in a comprehensive analysis of the effects. When this was accomplished, it was apparent that a systematic relationship exists between the xLD50 and changes in the plasma concentrations of 2-PAM. Lower challenge levels of GB or GD, e.g., $\leq$ 2.2 LD50, tend to decrease CP_{max} and AUC, whereas higher challenge levels of GB or GD, e.g., $\geq$ 2.8, tend to increase CP_{max} and AUC. At the extremes of challenge levels, the frequency of these changes is consistent, however; between the extremes, groups of animals demonstrate an intermediate response (see Fig. 3). In fact, the influence of GB and GD on plasma levels of 2-PAM relative to control levels was highly correlated (see Fig. 4 and 5).

A number of pathophysiological changes may account for the observed effects of nerve agents on 2-PAM levels. These changes include inhibition of metabolic enzymes, modification of renal elimination mechanisms and alterations in blood flow rates and/or patterns. Explanations of the pathophysiological changes will be complicated not only by the extent and diversity of these effects of the nerve agents themselves on the various physiological systems but also by the interactions between the therapeutic compounds, 2-PAM and atropine, and the nerve agents. Although one cannot ascribe specific physiological causes to the influence of nerve agents on 2-PAM plasma levels, a reasonable explanation of this phenomenon may be that the volume of distribution for 2-PAM is decreased following severe intoxication ($\geq$ 2.8 LD50). This may be a consequence of changes in the distribution and rate of blood flow to various organs, particularly those organs of elimination. Various authors (12-14) have reported significant changes in the pattern of blood flow after severe intoxication with organophosphorus inhibitors of cholinesterase. At the other end, less severe or moderate poisoning ($\leq$ 2.2 LD50) by nerve agents may increase the volume of distribution, perhaps by stimulating blood flow to various organ systems. For example, blood flow may increase to skeletal muscles at low levels of intoxication, through direct or indirect effects, increasing the volume of distribution of 2-PAM, but at high levels of intoxication, cardiovascular shock may supervene decreasing the volume of distribution.

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COMPARATIVE ABSORPTION OF INHALED AND INTRAMUSCULAR ATROPINE SULFATE

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ABSTRACT

The inhalation of atropine sulfate was investigated in an open-label, randomized 4 period rising dose study. Atropine sulfate 2, 4 and 6 mg by inhalation and 2 mg by IM injection were given to 8 healthy subjects. Serum levels from four subjects were monitored over 8 hr by RIA. Peak levels (mean ± S.D.) were 10.7 ± 3.9, 14.9 ± 5.9 and 17.5 ±2.5 ng/ml for the 2, 4 and 6 mg inhaled doses, respectively. The IM dose gave a peak level of 11.6 ± 1.6 ng/ml. The peak time for each dose was similar. The mean AUC for 2, 4, and 6 mg inhaled doses were 46.5 ± 13.0, 60.0 ± 23.1 and 80.6 ± 19.2 ng·hr/ml, respectively. For the IM dose the AUC was 52.9 ±3.8 ng·hr/ml resulting in a mean AUC ratio of the 2 mg inhaled dose to the 2 mg IM dose of 0.89 ± 0.28. Typical anticholinergic effects were seen after inhalation and IM dosing. Measurements of pulmonary function, ocular changes and vital signs were made during the 8 hr postdrug period and compared with the pharmacokinetic results. This study demonstrated systemic absorption of ascending doses of atropine sulfate after inhalation producing measurable blood levels and anticholinergic effects.

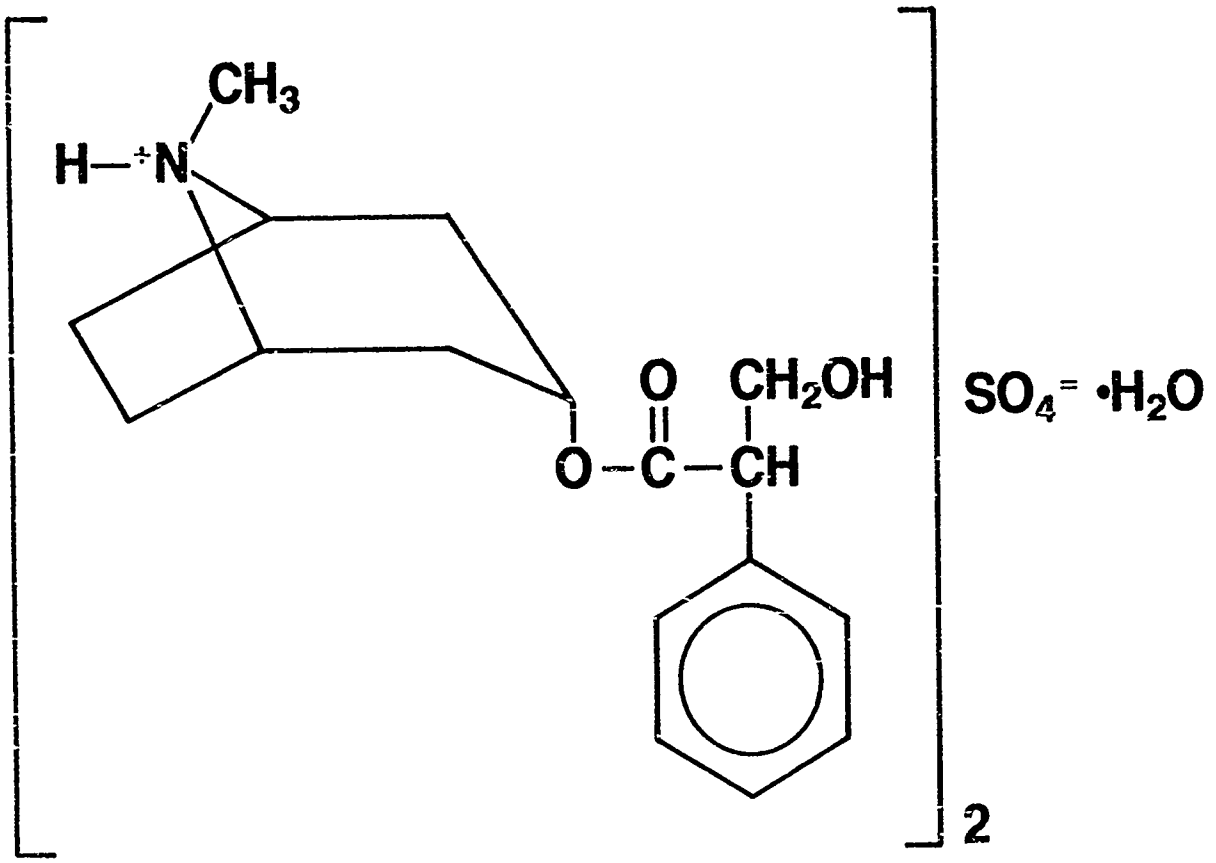
This work was supported by a grant from the U.S. Army Medical Research and Development Command under contract No. DAMD17-83-C-3141.

INTRODUCTION

Atropine, a naturally occurring belladonna alkaloid, is a potent anticholinergic agent often used as a component of general anesthesia to prevent the undesired side effects of cholinergic stimulation. Atropine has also been shown to cause improvement of air flow in chronic bronchitis, perennial childhood asthma, and cystic fibrosis when given by the aerosol route. Little is known about the systemic absorption of atropine sulfate through the lungs due to the lack of suitable bioanalytical methods to determine the level of drug in biological fluids.

This study investigated the feasibility of delivering atropine sulfate via oral inhalation by characterizing the oral absorption of drug following nebulized doses of 2, 4, and 6 mg.

CHEMISTRY



Atropine Sulfate

Benzeneacetic acid, ∝ — (hydroxymethyl) —, 8-methyl-8-azabicyclo — [3.2.1] oct-3-yl ester, endo — (±) —, sulfate (2:1) (salt), monohydrate.

METHODS

Subjects

Eight healthy nonsmoking adult males received single doses of 2, 4, and 6 mg of atropine sulfate by oral inhalation, or 2 mg of atropine sulfate by intramuscular (IM) injection in a randomized 4-period modified crossover trial with 46 to 48 hour washout periods between doses. No food or beverage was ingested for at least 2 hours prior to and following drug administration; water was allowed ad libitum throughout the study.

Drug Administration

The 2 mg IM dose was administered to the back of the thigh using commercially available Atropen® Autoinjectors. The inhalation doses were administered via nebulization of an aqueous solution. Each nebulized puff contained 0.5 ± 0.05 mg of drug, and was delivered over approximately 2 seconds with time allowed between puffs. The mean total duration of delivery of the 2, 4, and 6 mg nebulized doses were 1.93, 3.95, and 6.47 minutes, respectively.

Study Evaluations

Pharmacologic measurements, such as near point of accommodation, pupil size, pulmonary function, vital signs, and ECG intervals were evaluated following each single-dose treatment. Blood samples were obtained predose and periodically during the 8 hours following each dose.

Assay

The radioimmunoassay method of Wurzburger et al (J Pharmacol Exp Ther 1977; 203:435-441), using ^{14}C-atropine sulfate, was performed at Walter Reed Army Institute for Research.

Table 1. Plasma Kinetics Following Single Doses of Atropine Sulfate

Subject Number	Time To Peak Conc (hr)				Peak Conc (ng/ml)			
	IM, mg	Inhaled,mg			IM, mg	Inhaled, mg		
	2	2	4	6	2	2	4	6
1	a	0.5	0.5	0.5	10.6	13.5	14.0	21.0
2	2.0	1.0	2.0	0.5	10.5	5.0	7.4	15.4
3	1.0	0.5	0.5	1.0	11.5	11.1	16.8	16.2
4	1.0	2.0	0.5	1.0	13.9	13.1	21.4	17.4
Mean	1.3	1.0	0.9	0.8	11.6	10.7	14.9	17.5
SD	0.6	0.7	0.8	0.3	1.6	3.9	5.9	2.5
CV (%)	50	70	90	40	14	36	40	14

[a] Could not be determined since the peak level occured at 1.0 and 4 hours.

Table 2. Area-Under-The-Serum-Atropine Sulfate-Concentration-Versus-Time-Curves (AUCs) From Time Zero to Eight Hours

Subject Number	AUC (ng·hr/ml)				AUC Ratio
	IM, mg	Inhaled, mg			2 mg Inhaled
	2	2	4	6	2 mg IM
1	51.2	63.1	68.5	98.3	1.23
2	54.1	31.4	26.3	53.6	0.58
3	57.6	44.4	66.0	81.8	0.78
4	48.7	46.9	79.0	88.8	0.96
Mean	52.9	46.5	60.0	80.6	0.89
SD	3.8	13.0	23.1	19.2	0.28
CV (%)	7.2	28.0	38.6	23.8	31

Table 3. Incidence of Anticholinergic Effects

	Dose			
	Inhalation			Intramuscular
Anticholinergic Effects	2 mg	4 mg	6 mg	2 mg (IM)
Dry Mouth	6	6	6	7
Excessive Thirst	5	4	4	4
Blurred Vision	1	2	3	0
Difficulty Swallowing	0	3	5	1
Difficulty in Micturition	0	2	3	2
Facial Flushing	0	0	0	1
Hyperpyrexia or Dry Hot Skin	0	0	0	1
Nausea	0	1	3	0
Vomiting	0	0	2	0
Palpitations	0	0	1	0
Restlessness	1	0	0	0
Irritability	1	0	0	0
Confusion/Disorientation	2	0	0	0
Total No. Effects Reported	16	18	27	16
No. Subjects Reporting at Least One Effect	6	6	7	7
Total No. Subjects Receiving Dose	8	8	8	8

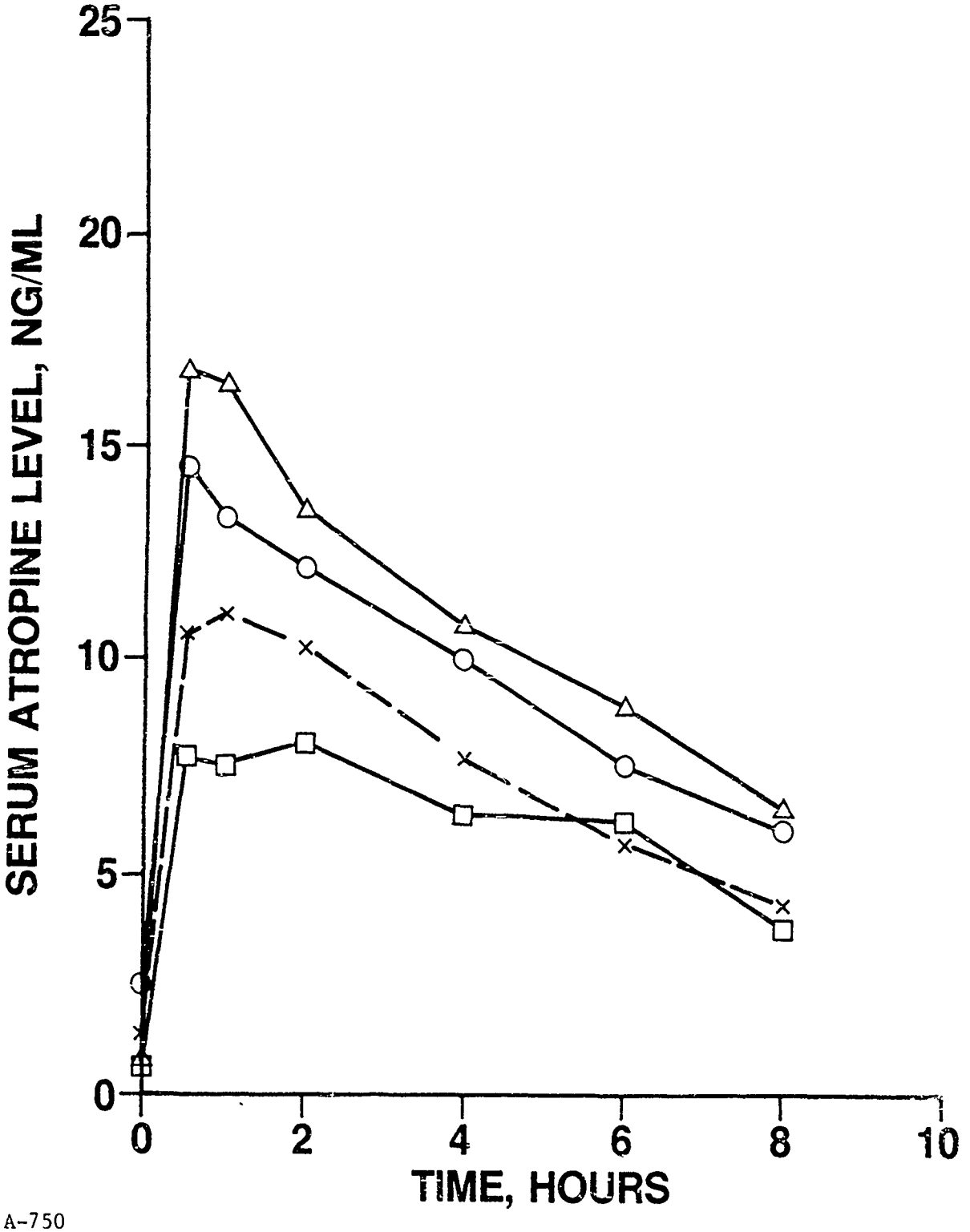
Table 4. Significant Pharmacologic Effects Following Atropine Sulfate Administration[a]

Evaluation	Effect at Dose of (mg)	Mean Baseline	Maximum Increase		
			Mean ± SD	Dose (mg)	Hours Postdose
Pulmonary					
FVC (Liters)	2 (IM)	4.92	0.125 ± 0.294	2 (IM)	2
FEV_1 (Liters)	All	3.92	0.406 ± 0.170	6	4
Visual					
Near Point of Accommodation [Both Eyes] (cm)	4, 6	10.3	6.26 ± 6.34	6	4
Pupil Size (mm)					
OD	All	3.2	1.77 ± 0.72	4	8
OS	All	3.2	1.67 ± 0.77	4	8
Vital Signs					
Heart Rate (BPM)	All	76.9	20.2 ± 25.5	2	0.5
Respiratory Rate (Per Min)	2	16.5	2.8 ± 2.1	2	1
Diastolic Blood Pressure (mm Hg)	2 (IM), 4, 6	74.2	13.8 ± 6.6	4	0.5
QT [Not Corr] (Sec)	2 (IM), 6	0.35	0.029 ± 0.030[b]	2 (IM)	8

[a] Wilcoxon Signed-Rank Test, $P<0.01$.
[b] Wilcoxon Signed-Rant Test, $P<0.05$.

Figure 1.

Mean Serum Concentrations of Atropine Sulfate in Four Subjects Given Inhaled Doses of 2 mg (□), 4 mg (○), and 6 mg (△), and an Intramuscular Injection of 2 mg (X).



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CONCLUSIONS

1. Systemic absorption of atropine sulfate following oral inhalation was demonstrated for the 4 subjects whose sera were analyzed for atropine sulfate. The mean plasma drug levels from the 2 mg IM dose were between those from the 2 and 4 mg inhaled doses.
2. Dose proportionality of the extent of absorption (AUCs) of the inhaled doses could not be determined due to insufficient blood sampling.
3. Atropine sulfate produced the expected anticholinergic effects, and very few other possibly drug-related, mild adverse experiences. The incidence of anticholinergic effects following the 2 mg IM dose was the same as that following the 2 mg inhaled dose. Increased incidence of anticholinergic effects with increasing doses of inhaled drug was observed.
4. When compared to predose values, statistically significant pharmacologic effects were observed, but none of the changes presented safety problems.
5. This study suggests that oral inhalation is a viable method of administering atropine sulfate.

A COMPARISON OF THE BIODISPOSITION OF DIISOPROPYLFLUOROPHOSPHATE IN MICE FOLLOWING INTRAVENOUS ADMINISTRATION AND INHALATION EXPOSURE

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ABSTRACT

The biological fate of diisopropylfluorophosphate (DFP) involves phosphorylation of proteins as well as its inactivation by spontaneous or enzymatic hydrolysis. The rapidity of these reactions suggests that the route of administration may dramatically alter the tissue disposition and metabolism of DFP. Studies were carried out to determine whether the biological fate of ^{3}H-DFP after i.v. injection was similar to that after inhalation. ^{3}H-DFP was administered i.v. (1 mg/kg) and 5 mice were decapitated after 1,5,15,30,60,120, 240,480 min and 1,3 and 7 days. For the disposition after inhalation, six mice were placed in individual animal restrainers which were attached to an exposure manifold so that the animals could breathe vapors from the volatilization of 4 mg of ^{3}H-DFP for 5 min. This exposure resulted in a ^{3}H-DFP dose of 2.1 mg/kg. Six mice were decapitated after 5,15,30,60,240,480 min and 1 day. Tissues and plasma from all mice were homogenized so that ^{3}H-DFP, free ^{3}H-diisopropylphosphoric acid (DIP), bound ^{3}H-DIP and non-extractable radioactivity could be separated by solvent extraction for quantitation by liquid scintillation spectrometry.

The time course of ^{3}H-DFP from 5 min to 1 day after either i.v. or inhalation showed that it rapidly penetrated all tissues and was quickly hydrolyzed to free ^{3}H-DIP or was covalently bound to tissues in the form of ^{3}H-DIP. The tissue levels of ^{3}H-DFP declined so rapidly that the levels of free ^{3}H-DIP actually exceeded those of ^{3}H-DFP for all tissues except for brain and liver at the very early time points. By 1 hr the greatest portion of the total radioactivity was in the form of bound ^{3}H-DIP regardless of route of administration. The concentrations of bound and free ^{3}H-DIP in lung were no greater after inhalation than after i.v. administration. The greatest difference between the two routes of administration appeared to be higher concentrations of bound and free ^{3}H-DIP in plasma following i.v. injection than after inhalation. Conversely, liver and kidney contained higher concentrations of free ^{3}H-DIP at the early times following inhalation as compared to i.v. administration.

The time course of cholinesterase inhibition in plasma, diaphragm and brain following i.v. injection of DFP was very similar to that following inhalation. Cholinesterase activity in all tissues had recovered by 7 days after both treatments, except for brain after inhalation exposure. In addition, the effects of DFP on motor function were similar after both routes of administration with complete recovery by 6-10 hr after treatment. In general, the biological fate, as well as the pharmacological effects, of DFP after i.v. administration were similar to those of DFP after inhalation exposure.

Supported by U.S.A.M.R.D.C. Contract DAMD17-82-C-2212.

INTRODUCTION

DIISOPROPYLFLUOROPHOSPHATE (DFP) UNDERGOES TWO BIOLOGICAL FATES UPON ENTERING TISSUES. DFP CAN BE INACTIVATED THROUGH SPONTANEOUS OR ENZYMATIC HYDROLYSIS TO DIISOPROPYLPHOSPHORIC ACID (FREE DIP). ALTERNATIVELY, DFP CAN FORM COVALENT BONDS WITH PROTEIN, RESULTING IN BOUND DIISOPROPYLPHOSPHORIC ACID (BOUND DIP). FREE DIP LACKS DFP-LIKE ACTIVITY, UNLIKE BOUND DIP, WHICH IS FORMED FROM DFP BINDING TO SPECIFIC ESTERASES (SUCH AS CHOLINESTERASE), NON-SPECIFIC ESTERASES, AND PROTEINS, AND THUS REPRESENTS POSSIBLE PHARMACOLOGICAL EFFECTS. THE RAPIDITY IN WHICH DFP UNDERGOES THESE REACTIONS SUGGESTS THE IMPORTANCE OF THE ROUTE OF ADMINISTRATION IN DETERMINING ITS BIODISPOSITION. THEREFORE, STUDIES WERE DONE TO COMPARE THE TISSUE CONCENTRATIONS OF THE PARENT MOLECULE FREE [^{3}H]DFP, PLUS THE TISSUE CONCENTRATIONS OF TWO METABOLITES FREE AND BOUND [^{3}H]DIP, AFTER I.V. ADMINISTRATION WITH THOSE TISSUE CONCENTRATIONS AFTER INHALATION EXPOSURE. IN ORDER TO EXAMINE THE EFFECT THAT THE ROUTE OF ADMINISTRATION HAD ON DFP'S PHARMACOLOGICAL EFFECTS, THE TIME COURSE OF CHOLINESTERASE INHIBITION IN SEVERAL TISSUES AND THE TIME COURSE OF DECREASED MOTOR FUNCTION WERE DETERMINED AFTER I.V. ADMINISTRATION AND THEN COMPARED TO THOSE EFFECTS SEEN AFTER INHALATION EXPOSURE.

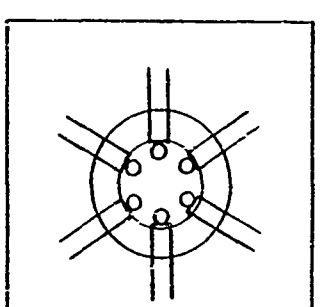
METHODS

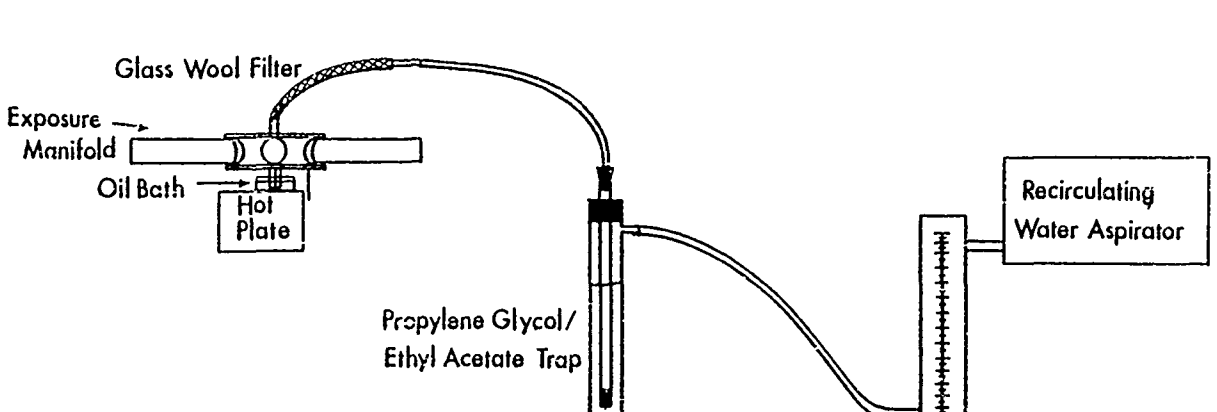
IN ORDER TO DETERMINE DFP'S BIODISPOSITION, ^{3}H-DFP WAS ADMINISTERED I.V. (1 MG/KG) AND 5 MICE WERE DECAPITATED AT EACH OF THE FOLLOWING TIME POINTS: 1, 5, 15, 30, 60, 120, 240, 480 MIN AND 1, 3 AND 7 DAYS. FOR THE DISPOSITION AFTER INHALATION, SIX MICE WERE PLACED IN INDIVIDUAL ANIMAL RESTRAINERS WHICH WERE ATTACHED TO THE EXPOSURE MANIFOLD SUCH THAT THE MICE COULD BREATHE THE VAPORS FROM THE VOLATILIZATION OF 4 MG OF ^{3}H-DFP FOR 5 MIN (FIGURE 1). THIS EXPOSURE RESULTED IN A DOSE OF 2.1 MG/KG. SIX MICE W RE DECAPITATED AT EACH OF THE FOLLOWING TIME POINTS: 5, 15, 30, 60, 240, 480 MIN AND 1 DAY. THE BRAIN, LIVER, LUNG, KIDNEY, DIAPHRAGM AND PLASMA WERE COLLECTED AND HOMOGENIZED SO THAT ^{3}H-DFP, FREE ^{3}H-DIP, BOUND ^{3}H-DIP, AND NON-EXTRACTABLE RADIOACTIVITY COULD BE SEPARATED BY SOLVENT EXTRACTION FOR QUANTITATION BY LIQUID SCINTILLATION SPECTROMETRY.

MICE WERE SIMILARLY TREATED WITH DFP BY I.V. OR INHALATION ADMINISTRATION AND THEN DECAPITATED AT TIME POINTS RANGING FROM 1 MIN TO 7 DAYS IN ORDER TO DETERMINE THE TIME COURSE OF CHOLINESTERASE INHIBITION. CONTROL ANIMALS WERE TREATED WITH SALINE FOR THE I.V. ADMINISTRATION OR VEHICLE FOR THE INHALATION EXPOSURE. THE BRAIN, DIAPHRAGM, AND PLASMA WERE COLLECTED FROM THE TREATED ANIMALS AND CHOLINESTERASE ACTIVITY WAS DETERMINED BY THE METHOD OF SIAKOTOS ET AL.

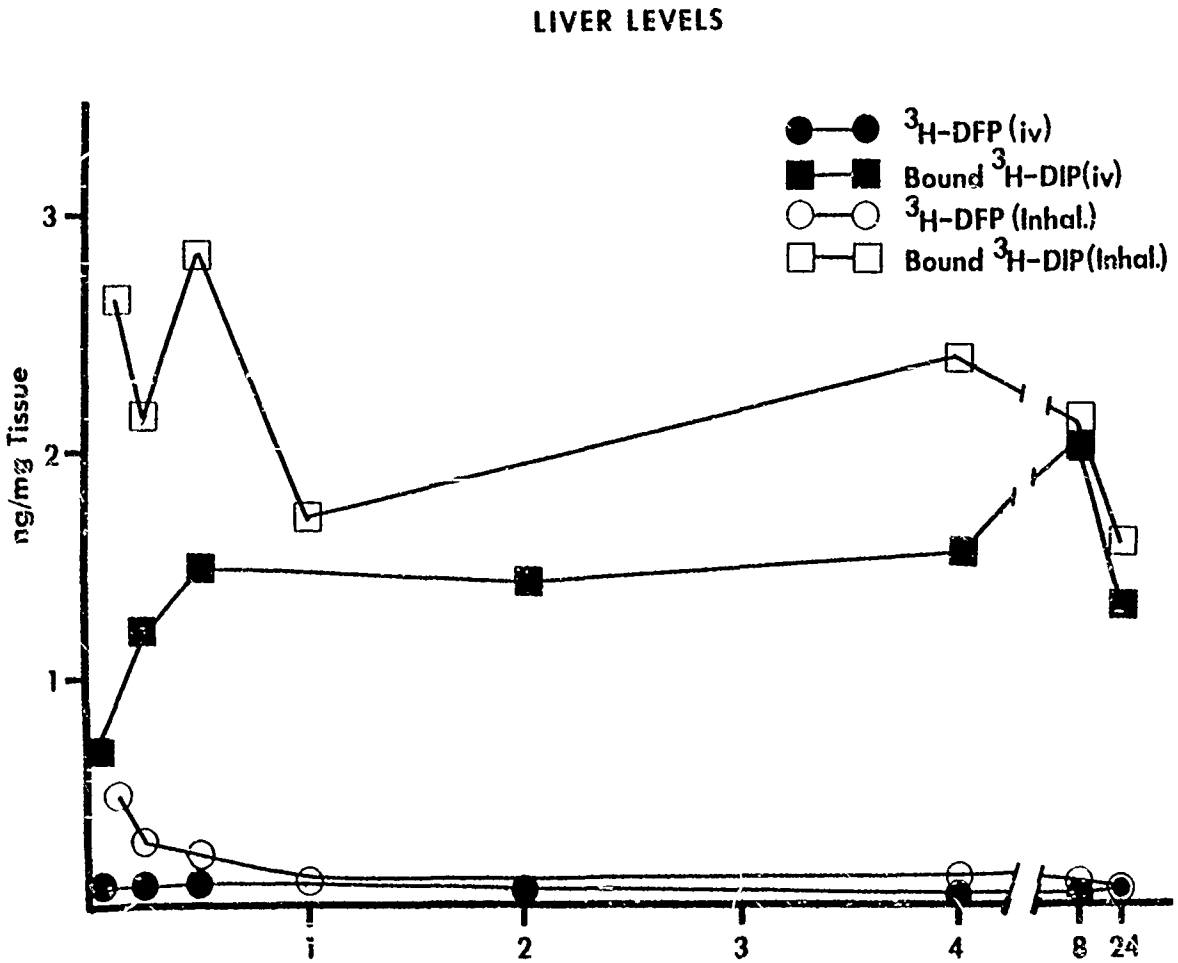
DFP-INDUCED REDUCTION IN MOTOR FUNCTION AFTER I.V. ADMINISTRATION WAS DETERMINED BY MEASUREMENT OF SPONTANEOUS ACTIVITY. GROUPS OF SIX MICE WERE INDIVIDUALLY PLACED IN ACTIVITY CHAMBERS AND INTERRUPTIONS OF A PHOTOCELL WERE RECORDED FOR 10 MIN. GROUPS OF MICE WERE TESTED EITHER IMMEDIATELY OR AT 20, 50, 110, 230 OR 350 MIN AFTER TREATMENT. MOTOR FUNCTION WAS ASSESSED AFTER DFP INHALATION BY MEASUREMENT OF MOTOR COORDINATION. MICE WERE TRAINED TO STAY ON A ROTATING ROD (11 RPM) FOR 1 MIN. AT THE APPROPRIATE TIME EXPOSED MICE THEN TESTED FOR THEIR ABILITY TO REMAIN ON THE ROTATING ROD FOR ONE MINUTE. THE SIX MICE PER TIME POINT WERE TESTED AT 5, 30, 60, 120, 240, 360 AND 600 MIN. CONTROL MICE FOR BOTH MEASURES OF MOTOR FUNCTION WERE HANDLED IN THE APPROPRIATE WAY.

INHALATION APPARATUS





LIVER LEVELS



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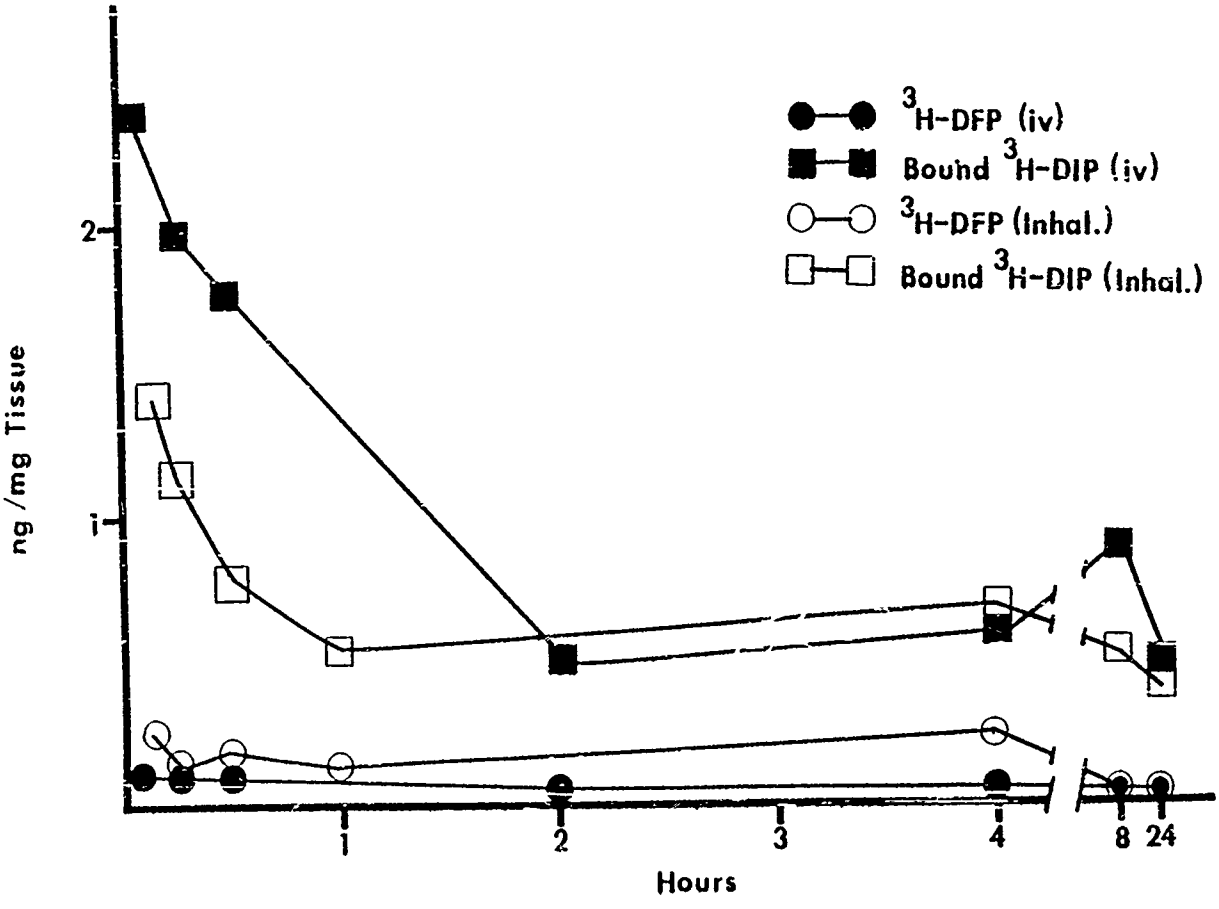
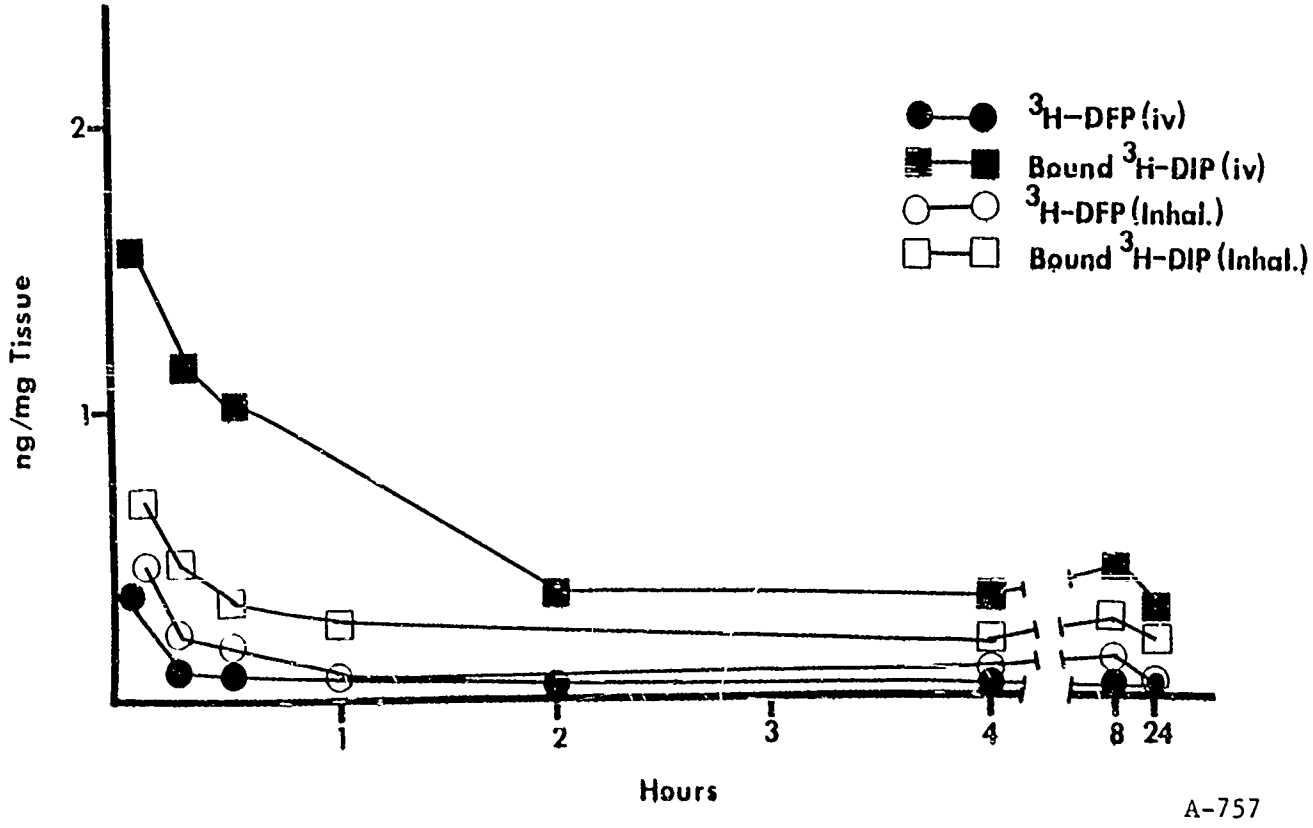
PLASMA LEVELS



LUNG LEVELS



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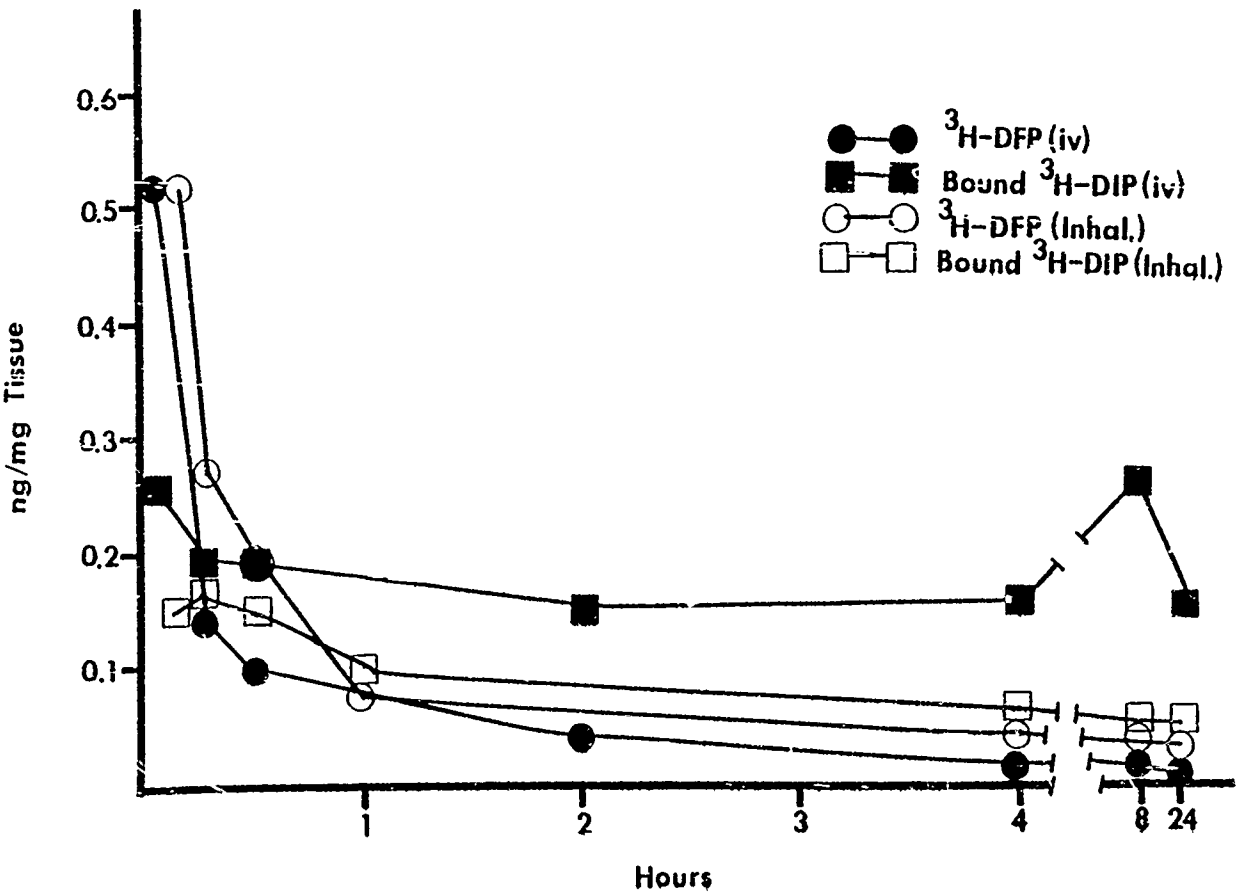
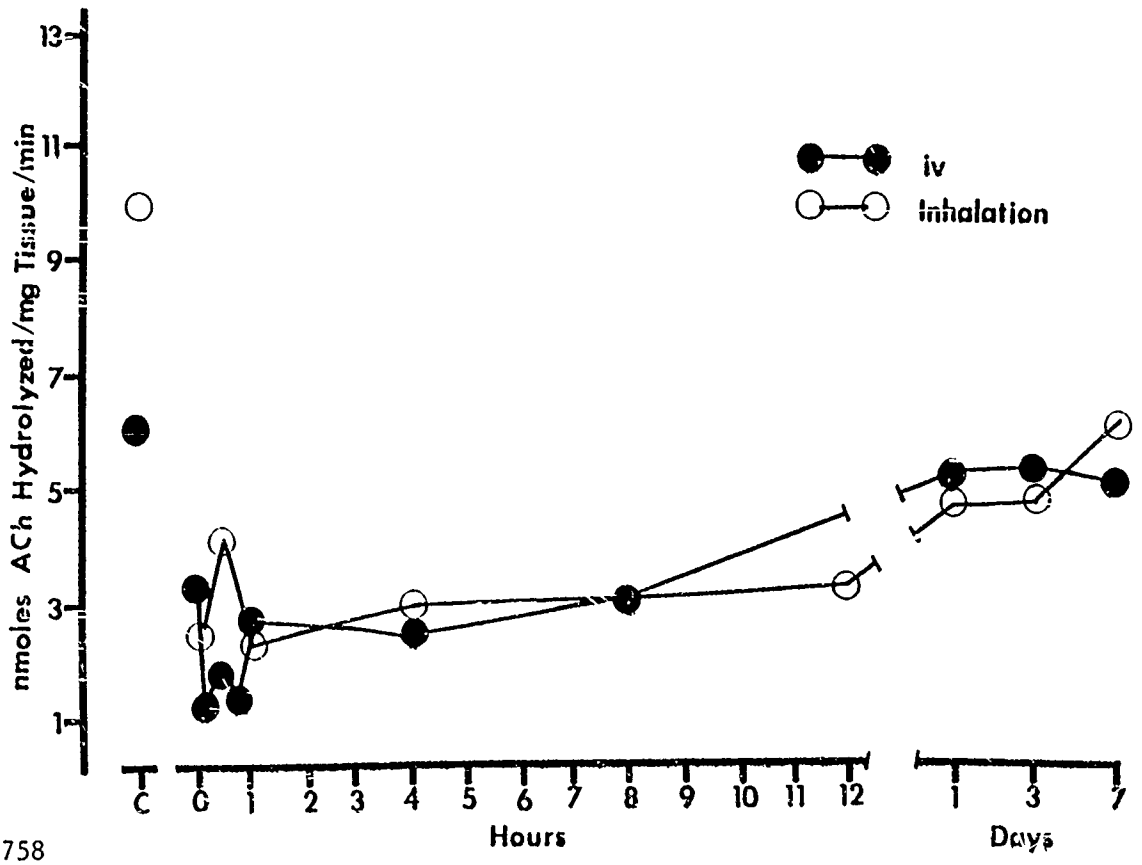
BRAIN LEVELS



BRAIN CHOLINESTERASE ACTIVITY



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TIME COURSE OF HYPOACTIVITY AS MEASURED BY SPONTANEOUS ACTIVITY FOLLOWING I.V. ADMINISTRATION OF DFP (1 MG/KG)

	INTERRUPTIONS OF THE PHOTOCELL BEAM	
TIME	CONTROL	DFP
10 MIN	83 ± 5.2	41 ± 6
30 MIN	94 ± 33	42 ± 10
1 HR	100 ± 8.4	58 ± 15
2 HR	98 ± 10	50 ± 5
4 HR	101 ± 25	74 ± 8
6 HR	91 ± 8	90 ± 12

TIME COURSE OF MOTOR COORDINATION AS MEASURED BY ROTAROD FOLLOWING ADMINISTRATION OF DFP[B]

	PERCENT THAT FELL OFF[A]	
TIME	CONTROL	DFP
5 MIN	29 ± 10	65 ± 7
30 MIN	17 ± 10	74 ± 4
1 HR	12 ± 8	66 ± 11
2 HR	25 ± 5	62 ± 17
4 HR	12 ± 8	48 ± 21
6 HR	4 ± 4	36 ± 13
10 HR	12 ± 12	26 ± 4

[A] PERCENTAGE OF THOSE ANIMALS THAT FAILED TO COMPLETE THE TASK, I.E., THOSE ANIMALS THAT FELL OFF THE ROTATING ROD WITHIN 1 MINUTE. THE MEANS ± S.E. FOR 4 EXPERIMENTS ARE PRESENTED. EACH GROUP CONSISTS OF 6 MICE. THE SAME GROUP OF MICE WAS TESTED AT EACH TIME POINT FOR A PARTICULAR EXPERIMENT.

[B] EXPOSURE BY INHALATION TO 4.0 MG DFP FOR 5 MINUTES.

SUMMARY

1. ^{3}H-DFP RAPIDLY PENETRATED ALL TISSUES WHERE IT WAS QUICKLY HYDROLYZED OR COVALENTLY BOUND AFTER EITHER I.V. OR INHALATION ADMINISTRATION.
2. BY 1 HR, THE GREATEST PORTION OF TOTAL RADIOACTIVITY IN ALL TISSUES WAS IN THE FORM OF BOUND ^{3}H-DIP, REGARDLESS OF THE ROUTE OF ADMINISTRATION.
3. THE CONCENTRATIONS OF BOUND AND FREE ^{3}H-DIP IN LUNG WERE UNEXPECTEDLY NOT GREATER FOR INHALATION THAN I.V. ADMINISTRATION.
4. THE GREATEST DIFFERENCE WAS FOUND IN THE HIGHER LEVELS OF BOUND ^{3}H-DIP IN THE PLASMA AFTER I.V. INJECTION THAN AFTER INHALATION EXPOSURE.
5. CHOLINESTERASE ACTIVITY IN THE BRAIN, PLASMA AND DIAPHRAGM WAS RAPIDLY AND PROFOUNDLY DEPRESSED AND REMAINED SO FOR ABOUT 1 DAY.
6. THE TIME COURSE OF CHOLINESTERASE INHIBITION FOR ALL THREE TISSUES WAS VERY SIMILAR REGARDLESS OF THE ROUTE OF ADMINISTRATION.
7. ALTHOUGH MOTOR FUNCTION WAS NOT DETERMINED BY THE SAME MEASUREMENT FOR BOTH ROUTES OF ADMINISTRATION, SIMILAR TIME COURSE PROFILES INDICATE S..ILAR EFFECTS REGARDLESS OF THE ROUTE.

CONCLUSION

GREATER DIFFERENCES IN THE BIODISPOSITION OF ^{3}H-DFP AFTER I.V. AND INHALATION ADMINISTRATION WOULD HAVE BEEN OBSERVED IF IT WERE NOT FOR THE FACT THAT ^{3}H-DFP DID NOT BIND EXTENSIVELY TO LUNG FOLLOWING INHALATION. THEREFORE, THE BIODISPOSITION AS WELL AS THE PHARMACOLOGICAL EFFECTS MEASURED IN THIS STUDY OF DFP AFTER I.V. ADMINISTRATION WERE SIMILAR TO THOSE OF DFP AFTER INHALATION EXPOSURE.

STABILIZATION, ANALYSIS AND TOXICOKINETICS OF THE FOUR STEREOISOMERS OF SOMAN IN RAT BLOOD

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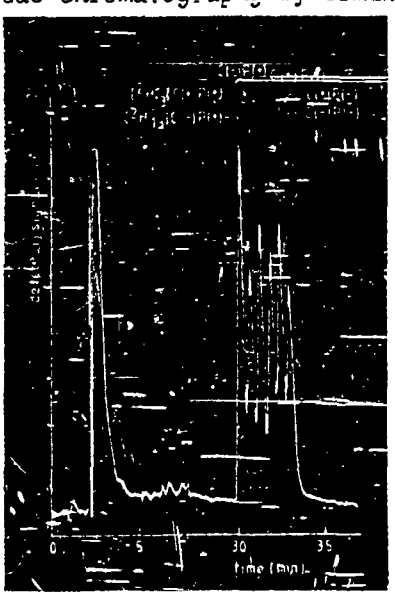
INTRODUCTION

Soman is much more persistent in vivo than previously assumed, which presents further complications for treatment of intoxications with this agent. For this reason, toxicokinetic studies of soman in various species are needed. Prior to this, methods have to be developed for the trace analysis of soman in blood and tissue. These methods should (i) deal with the stabilization of the highly reactive isomers during work-up, and (ii) distinguish between the four stereoisomers of soman in view of their widely differing toxicological properties.

ANALYTICAL PROCEDURE

Gas chromatography of soman-stereoisomers



C(±)P(±)-soman (50 pg) and the internal standard ($^2H_{13}$)-C(±)P(+)-soman on a Chirasil Val column (l=50 m; i.d. 0.50 mm), synthezized and coated in house. NP-detector (attn. 8). The internal standard was obtained from C(±)P(±)-($^2H_{13}$-pinacolyl) methylphosphonofluoridate by selective removal of the P(-)-isomers with α-chymotrypsin.

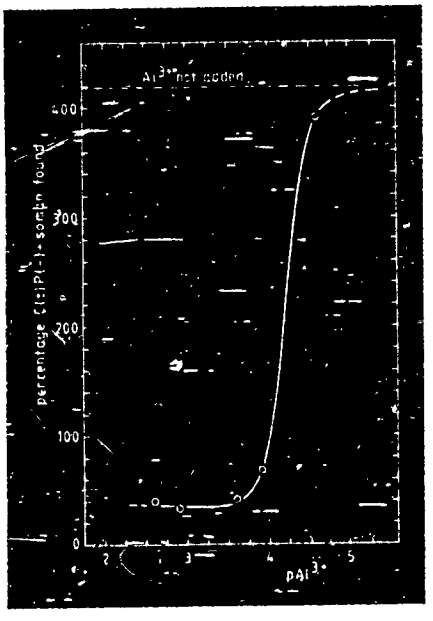
Stabilization of C(±)P(+)-soman

- In order to occupy binding sites, blood was used from respirated rats, to which soman (1 LD_{50}, i.v.) had been administered one hour earlier.
- The hydrolytic activity of phosphorylphosphatases was blocked by acidification of blood samples with acetate buffer to final pH 4.2 at 0°C.
- After addition of 10 ng soman/ml blood, the concentration of the isomers was measured 5 and 45 min later.

Incubation time (min)	Isomer found as percentage of added amount				
	C(-)P(+)	C(+)P(+)	C(+)P(-)	C(-)P(-)	
5	98 ± 1	103 ± 3	304 ± 35	252 ± 26	(n=3)
45	92 ± 6	101 ± 2	1311 ± 44	1141 ± 73	(n=3)

- The P(+)-isomers are sufficiently stabilized at pH 4.2, 0°C.
- Rapid generation of P(-)-isomers is probably due to reactivation of soman-inhibited aliesterase by fluoride ions (De Jong c.s.). If so, complexation with aluminium ions may redress this complication.

Stabilization of C(±)P(-)-soman



- $Al^{3+} + F^- \rightleftharpoons AlF^{2+}$ ($K=10^{6.1}\ M^{-1}$).
- Blood from rats pretreated with 1 LD_{50} of soman, $[F^-]=15\ \mu M$, was mixed with acetate buffer. After addition of various $[Al^{3+}]$ and of 10 ng soman/ml blood, C(±)P(-)-soman was measured 40 min later.
- Under stabilized conditions ($pAl^{3+} < 4$), only 40% of added C(±)P(-)-soman is found, which may be due to incomplete "saturation" of binding sites by pretreatment with 1 LD_{50} of soman.
- In order to block residual binding sites, the soman-analogue neopentyl sarin, $Me_3CCH_2O(Me)P(O)F$, was added to the incubation mixture.

Soman isomer	Soman isomer found as percentage of added amount			
	Without neopentyl sarin		With neopentyl sarin	
	Incubation time (min)		Incubation time (min)	
	5	45	5	45
C(+)P(-)	87 ± 9	56 ± 36 (n=3)	100 ± 7	102 ± 6 (n=2)
C(-)P(-)	93 ± 7	54 ± 36 (n=3)	96 ± 1	98 ± 1 (n=2)

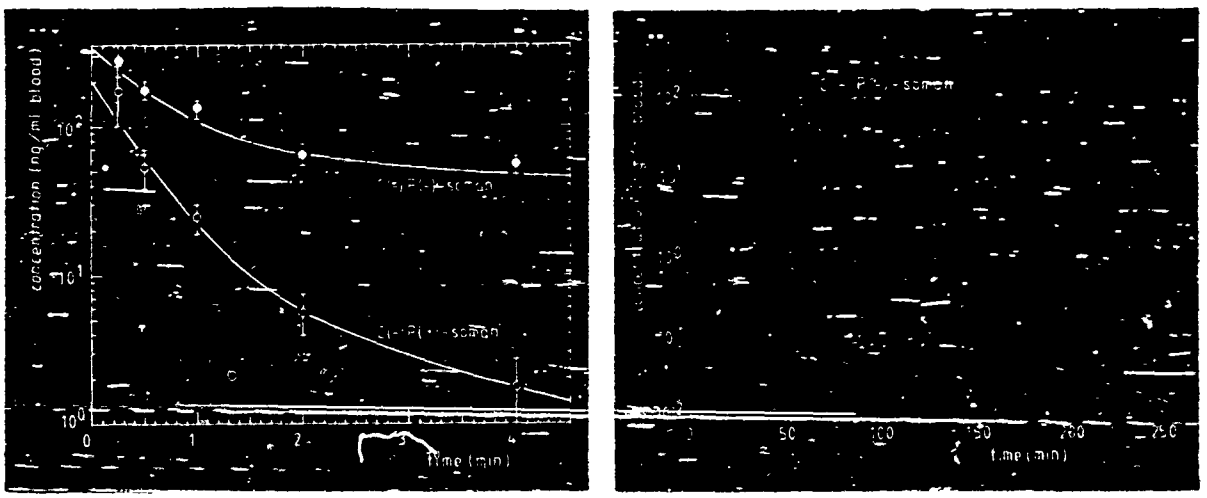
- Addition of neopentyl sarin, and of aluminium ions, results in complete stabilization of C(±)P(-)-soman at pH 4.2, 0°C.
- Rapid generation of C(±)P(-)-soman by uncomplexed fluoride ions leads to the measurement of artifactual blood levels of soman in rodents.

Summary of analytical procedure

- Stabilization of soman in rat blood samples by means of (i) acidification to pH 4.2 at 0°C, for stabilization C(±)P(+)-isomers, (ii) addition of Al^{3+} (2.5 mM) for complexation of F^-, which generate C(±)P(-)-soman from inhibited aliesterase, (iii) addition of neopentyl sarin in order to block covalent binding sites for C(±)P(-)-soman.
- Work-up via extraction with Sep-Pak C_{18}, followed by elution with ethyl acetate. Over all extraction recoveries 52 ± 8%.
- Gas chromatographic analysis on a wide bore Chirasil Val column, with alkali flame ionization detection. Limit of detection: ca. 250 pg isomer per blood sample (ca. 0.5 nM).

TOXICOKINETICS OF SOMAN IN RATS

- Rats were anaesthetized with sodium (hexo)barbital (i.p.). After installation of a carotid cannula, the animals received atropine sulfate (50 mg/kg, i.p.), 5 min before 6 LD_{50} of C(±)P(±)-soman was administered i.v. (495 µg/kg). The rats were kept alive with artificial respiration. Blood samples were taken via the carotid cannula.



Soman-isomer	$Conc. = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$					
	A^a	B^a	C^a	α^b	β^b	γ^b
C(+)P(+)[c]	-	-	-	-	-	-
C(-)P(+)	193	11	-	2.5	0.46	-
C(-)P(-)	263	70	1.0	1.8	0.10	0.017
C(+)P(-)	274	73	0.58	1.7	0.12	0.011

[a] $ng.ml^{-1}$ [b] min^{-1} [c] completely degraded within 0.25 min.

- Whereas the C(+)P(+)-isomer disappears completely from blood within 0.25 min, largely due to hydrolysis by phosphorylphosphatases, the C(-)P(+)-isomer survives sufficiently long to allow the observance of a bi-exponential decay of the concentration with time.
- The toxicokinetics of the two P(-)-isomers are similar, the decrease of the concentrations of both isomers obeying a tri-exponential function.
- Tentatively, the rapid elimination of P(-)-isomers in the first phase is ascribed to phosphonylation reactions in blood and tissue.
- The third, slow, phase for the P(-)-isomers corroborates the toxicological evidence for the in vivo persistence of soman, and is in accordance with the observed soman depot in rats, after administration of 6 LD_{50} of soman (i.v.).

7. Neurotransmitter Effects

POTENTIATION OF TOXICITY AND PREVENTION OF TOLERANCE TO PROLONGED DFP EXPOSURE

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ABSTRACT

Male Sprague-Dawley rats when treated with a single injection of diisopropylfluorophosphate (DFP; 1.5 mg/kg) exhibited symptoms of cholinergic hyperactivity. No tolerance developed to this dose of DFP. Rats injected with DFP (0.5 mg/kg, sc) daily showed symptoms at day three similar to those observed with a single injection of DFP (1.5 mg/kg). However, further administration of DFP (0.5 mg/kg) led to behavioral tolerance, as evidenced by the disappearance of symptoms following 7-8 days of injections.

The effects of both regimens of DFP on the activities of choline acetyltransferase (ChAT, E.C. 2.3.1.6.), acetylcholinesterase (AChE, E.C. 3.1.1.7.), butyrylcholinesterase (BuChE, E.C. 3.1.1.8.) in brain and in skeletal muscles and the nicotinic acetylcholine receptor (nAChR) binding in diaphragm muscle were determined.

Daily injection of DFP (0.5 mg/kg) for 5 days significantly inhibited ($>$80%) total AChE activity and its molecular forms in both brain and muscle. After 14 days of DFP injections AChE activity significantly ($p < 0.01$) recovered in muscles (24-54% inhibition) but not in brain. This might indirectly indicate de novo synthesis of AChE in muscles during the development of tolerance to DFP. Repeated DFP treatment (5 and 14 days) also resulted in a significant ($p < 0.05$) decrease of available ^{3}H-ACh

binding sites in solubilized membranes of diaphragm (46.7 ± 2.3 fmole/mg protein compared to control value of 112.3 ± 8.1 fmole/mg protein) without affecting the affinity constant. ChAT activity in diaphragm, soleus and EDL significantly ($p < 0.01$) increased after repeated DFP (0.5 mg/kg) injections, but not after single treatment. It is concluded that tolerance to DFP administration, depending on the tissue studied, may possibly include reduced nAChR binding sites and increased de novo synthesis of AChE.

A combined treatment of rats with BuChE inhibitors such as iso-OMPA (tetramonoisopropylpyrophosphortetramide; 3 mg/kg, sc) or mipafox (N,N'-diisopropylphosphorodiamidofluoridate; 0.05 mg/kg, sc) and DFP (0.5 mg/kg, sc) for 5 consecutive days potentiated the DFP toxicity including greater inhibition of AChE and BuChE in brain and muscle tissue when compared with the effects of DFP alone. In these concentrations iso-OMPA and mipafox alone significantly inhibited aliesterases while they had no effect on AChE activity; and, both completely abolished the development of tolerance to DFP. There was no recovery of AChE activity and no animal survived more than 5-7 days of treatment. The mechanism for this increased DFP toxicity may be due to the inhibition of an enzyme that hydrolyzes DFP by mipafox and iso-OMPA and/or to decrease in non AChE DFP binding sites such as BuChE, aliesterases and other proteins.

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INTRODUCTION

At the cholinergic synapse acetylcholinesterase (AChE) plays an important role in the removal of acetylcholine (ACh) from the synaptic cleft. Irreversible inhibitors of AChE such as diisopropylfluorophosphate (DFP), profoundly modify neuromuscular transmission as seen in twitch potentiation, fasciculation, muscular weakness and acute necrosis of muscle fibers. Tolerance

to organophosphates including DFP, is reported to develop to their behavioral and toxic effects when they are administered for a period of more than seven days.

Various cholinergic biochemical (functional mechanisms) changes underlying this phenomenon have been reported such as (a) reduced acetylcholine receptor (muscarinic and nicotinic) sites, (b) reduced choline uptake, (c) reduced ability of extracellular ACh to inhibit the release of ACh elicited by depolarization, and (d) stimulation of AChE synthesis in skeletal muscles. Other mechanisms leading to adaptation to low concentrations of DFP are unspecific binding to enzymes with serine active sites other than AChE such as aliesterases and butyrylcholinesterases and a variety of proteins which may reduce the free concentration of DFP otherwise available for anticholinesterase action and an enhanced rate of detoxication of DFP by elevated DFPase activity.

The objective of the present investigation was to study mechanisms involved in the development of tolerance to DFP, especially in skeletal muscles, and brain.

Pretreatment of rats with irreversible BuChE inhibitors (iso-OMPA and mipafox) which are known to bind to serine sites other than AChE and to inhibit DFPase reversibly was used to study the role of unspecific binding of DFP and of its hydrolysis in the development of adaptation.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats weighing about 200 g, housed in standard hygienic conditions, maintained on a 12 h light-dark cycle with food and water available ad libitum were used throughout these studies.

Drug administration: DFP (diisopropylfluorophosphate, 0.5 mg/kg), iso-OMPA (tetramonoisopropylpyrophosphortetramide, 3 mg/kg), and mipafox (N,N'-diisopropylphosphorodiamidofluoridate, 0.05 mg/kg), were daily prepared in normal saline in such a volume that the required dose was in 0.1 ml/100 g body weight and injected subcutaneously. In acute experiments, animals were

treated with a single sublethal dose of 1.5 mg DFP/kg; while for subchronic experiments, once daily for 5 or 14 consecutive days with a dose of 0.5 mg/kg. The BuChE inhibitors (iso-OMPA, 3 mg/kg or mipafox, 0.05 mg/kg) were administered alone or as pretreatment to DFP (30 min prior to DFP). A detailed schedule of drug administration to determine the effects on liver aliesterase is given in Table 8.

Collection of tissues: Rats were sacrificed by decapitation and the diaphragm, slow twitch soleus (SOL), and fast twitch extensor digitorum longus (EDL) muscles were dissected. Liver and discrete brain regions (cortex, striatum, stem and hippocampus) were quickly isolated on ice. All tissues, soon after their collection, were analyzed for the activity of AChE, BuChE and ChAT.

Biochemical analyses

Choline acetyltransferase: (ChAT, acetyl CoA choline o-acetyltransferase; E.C. 2.3.1.6.). The activity of ChAT was assayed in skeletal muscles employing the radicchemical method of Fonnum (1975).

Acetylcholinesterase and its molecular forms: (AChE, acetylcholine (acetyl-)hydrolase; E.C. 3.1.1.7.). The activity of total AChE in skeletal muscles and in discrete brain regions was assayed according to the method of Ellman et al. (1961) using acetylthiocholine iodide as the substrate. The different molecular weight forms of AChE were separated by velocity sedimentation on 5-20% linear sucrose density gradients following the modified methods of Massoulie et al. (1970) as described by Groswald and Dettbarn (1983) and were quantitated with the radiometric assay (Johnson and Russell, 1975). Proteins with known sedimentation constants, alkaline phosphatase (6.1S), catalase (11.1S), and B-galactosidase (16.0S) were added to the gradients for determination of the sedimentation constants of the AChE molecular forms.

Butyrylcholinesterase: (BuChE; E.C. 3.1.1.8.). The activity of BuChE was measured in skeletal muscles and brain employing the method of Ellman et al. (1961) using butyrylthiocholine iodide as the substrate.

Aliesterases: The activity of aliesterase (tributyrinase; E.C. 3.1.1.1.) was determined in freshly isolated liver using tributyrin as substrate employing Hestrin's technique (Hestrin, 1950) with slight modifications (Gupta et al., 1985c). Protein was determined by Lowry's method (1951) using bovine serum albumin as standard.

Nicotinic acetylcholine receptor binding (nAChR): The specific binding of ^{3}H-acetylcholine (^{3}H-ACh) in diaphragm was assayed employing the technique recently described for brain (Schwartz et al., 1982) modified for skeletal muscles (Gupta et al., 1985a,b). The specific binding was determined as the mean difference between the binding found with and without carbachol.

Data were subjected to statistical analysis employing unpaired two tailed Student's 't' test.

RESULTS

Clinical: Following repeated administration of DFP, 0.5 mg/kg/day, the onset of toxicity symptoms was evident after three doses. Maximum severity occurred after five doses, comparable to that caused by a single acute dosage (1.5 mg DFP/kg). Continued exposure for more than 7 days resulted in behavioral tolerance as evidenced by disappearance of toxicity signs and body weight gain. Rats pretreated daily with either of the BuChE inhibitors exhibited symptoms of DFP toxicity on day two and none of the rats survived more than 5 days.

Biochemical: Results presented in Tables 1 and 2 show that a single dose of DFP (0.5 mg/kg) caused significant ($p < 0.01$) inhibition of AChE in all four brain regions (54-69%) and in all three skeletal muscles ($> 30\%$) tested. Maximal inhibition (80%) was seen in the muscles as well as the brain regions examined after the fifth dose. When DFP was injected for two weeks, the activity of AChE in skeletal muscles had recovered significantly

TABLE 1. EFFECT OF DFP ADMINISTRATION (0.5 MG/KG, SC) FOLLOWING SINGLE AND REPEATED DOSES (5 AND 14 DAYS) ON ACETYLCHOLINESTERASE (ACHE) ACTIVITY IN BRAIN AND SKELETAL MUSCLES OF RAT

	AChE Activity			
Muscles	Control	1 Day	5 Days	14 Days
Diaphragm	78.3±1.6 (100)	53.6±2.5[a] (68)	16.9±3.0[a] (22)	35.9±1.7[a,b] (46)
Soleus	60.1±1.2 (100)	41.4±1.3[a] (69)	11.3±1.8[a] (19)	45.8±2.4[a,b] (76)
EDL	105.8±1.0 (100)	67.5±6.7[a] (64)	20.8±2.5[a] (20)	58.2±2.3[a,b] (55)
Brain Regions				
Cortex	271.2±4.0 (100)	84.2±4.0[a] (31)	27.8±5.1[a] (10)	15.8±1.2[a] (6)
Striatum	1610.4±31.6 (100)	687.6±33.3[a] (43)	142.1±4.3[a] (9)	151.2±19.2[a] (9)
Brain Stem	579.2±11.0 (100)	267.3±14.8[a] (46)	111.6±1.4[a] (19)	122.8±1.4[a] (21)
Hippocampus	335.8±8.8 (100)	120.2±9.4[a] (36)	53.4±1.4[a] (16)	59.3±2.0[a] (18)

Values are mean ± SEM of AChE activity (umole acetylthiocholine iodide/g/h) from 5-12 rats.
Values in parentheses indicate remaining AChE activity and expressed as % of control (100%).

[a] $p < 0.01$ when compared with the values of controls.
[b] $p < 0.01$ when values of day 14 compared with the values of day 5.

TABLE 2. EFFECT OF DFP ADMINISTRATION (0.5 MG/KG, SC) FOLLOWING SINGLE AND REPEATED DOSES (5 AND 14 DAYS) ON BUTYRYLCHOLINESTERASE (BUCHE) ACTIVITY IN BRAIN AND SKELETAL MUSCLES OF RAT

	BuChE Activity			
Muscles	Control	1 Day	5 Days	14 Days
Diaphragm	10.2±0.2 (100)	6.4±0.7[a] (63)	0.8±0.3[a] (8)	5.2±0.7[a,b] (51)
Soleus	10.3±0.3 (100)	8.2±0.2 (80)	2.4±1.2[a] (23)	6.4±0.9[b] (62)
EDL	9.7±0.8 (100)	8.4±0.8 (87)	4.7±1.2[a] (48)	5.8±1.0 (60)
Brain Regions				
Cortex	18.2±1.1 (100)	6.9±1.1[a] (38)	6.8±0.6[a] (37)	6.6±0.6[a] (36)
Striatum	20.3±0.5 (100)	7.5±1.1[a] (37)	6.6±0.3[a] (33)	6.6±0.6[a] (33)
Brain Stem	36.3±1.9 (100)	13.6±0.8[a] (37)	10.0±0.5[a] (28)	9.2±0.4[a] (25)
Hippocampus	16.4±0.4 (100)	8.2±0.5[a] (50)	7.1±0.3[a] (43)	7.6±0.5[a] (46)

Values are the mean ± SEM of BuChE activity (umole butyrylthiocholine iodide/g/h) from 5-12 rats.
Values in parentheses indicate remaining BuChE activity and expressed as % of control (100%).

[a] $p < 0.01$ when compared with the values of controls.
[b] $p < 0.05$ when values of day 14 compared with the values of day 5.

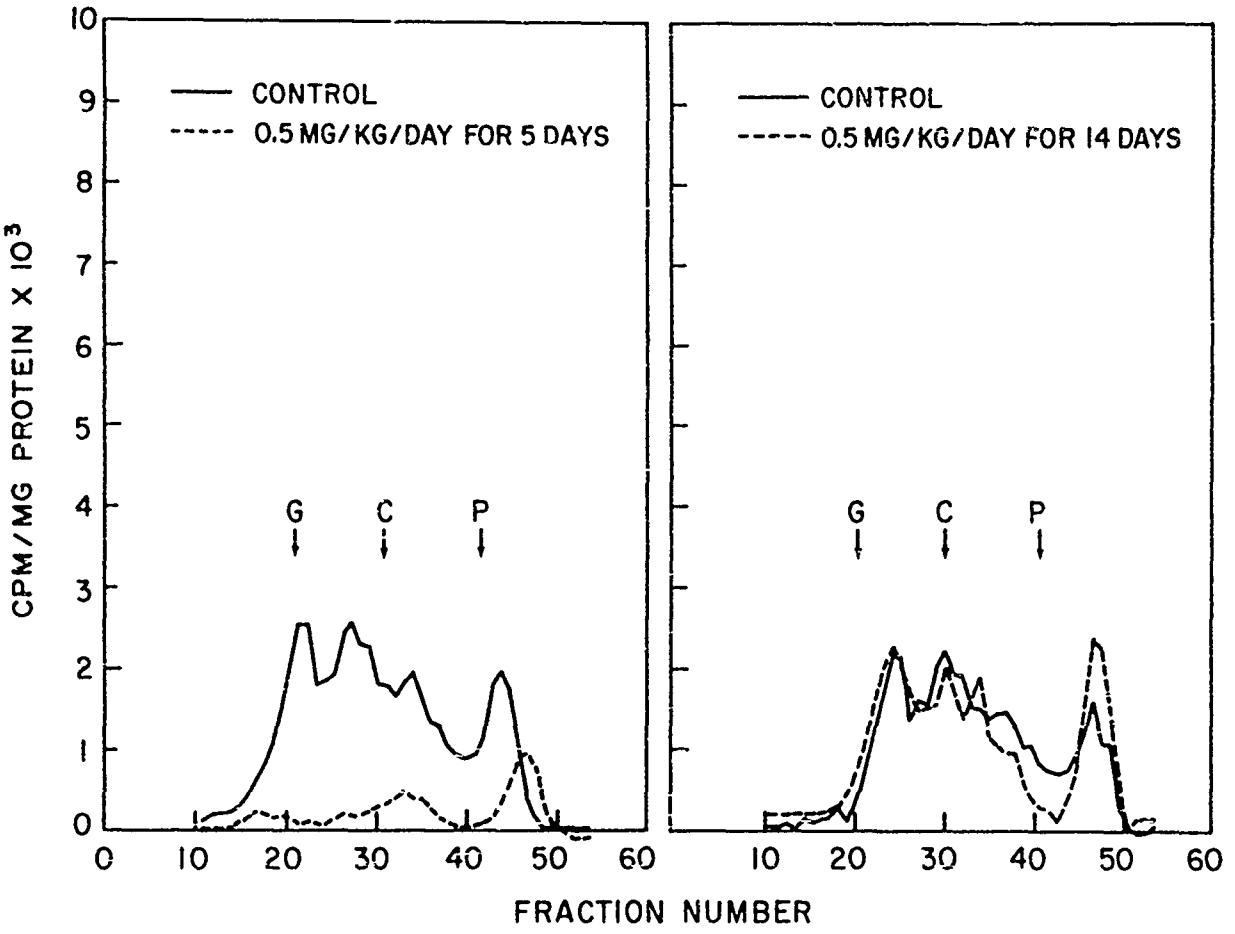

Fig. 1. Effects of DFP administered in repeated doses for 5 days (left) and 14 days (right) on acetylcholinesterase (AChE) molecular forms in soleus muscle.

Velocity sedimentation gradient separation of the AChE from soleus muscle: gradients contained 200 ug of a high speed supernatant from the muscle which was layered on a 5-20% sucrose gradient and centrifuged at 35,000 rpm for 18 hrs. Approximately 55 fractions were collected and 0.03 ml was incubated for four hours in the presence of ^{3}H-acetylcholine and iso-OMPA (10 uM). Arrows indicate position of markers, G = B-galactosidase (16.0S), C = catalase (11.1S), and P = alkaline phosphatase (6.1S).

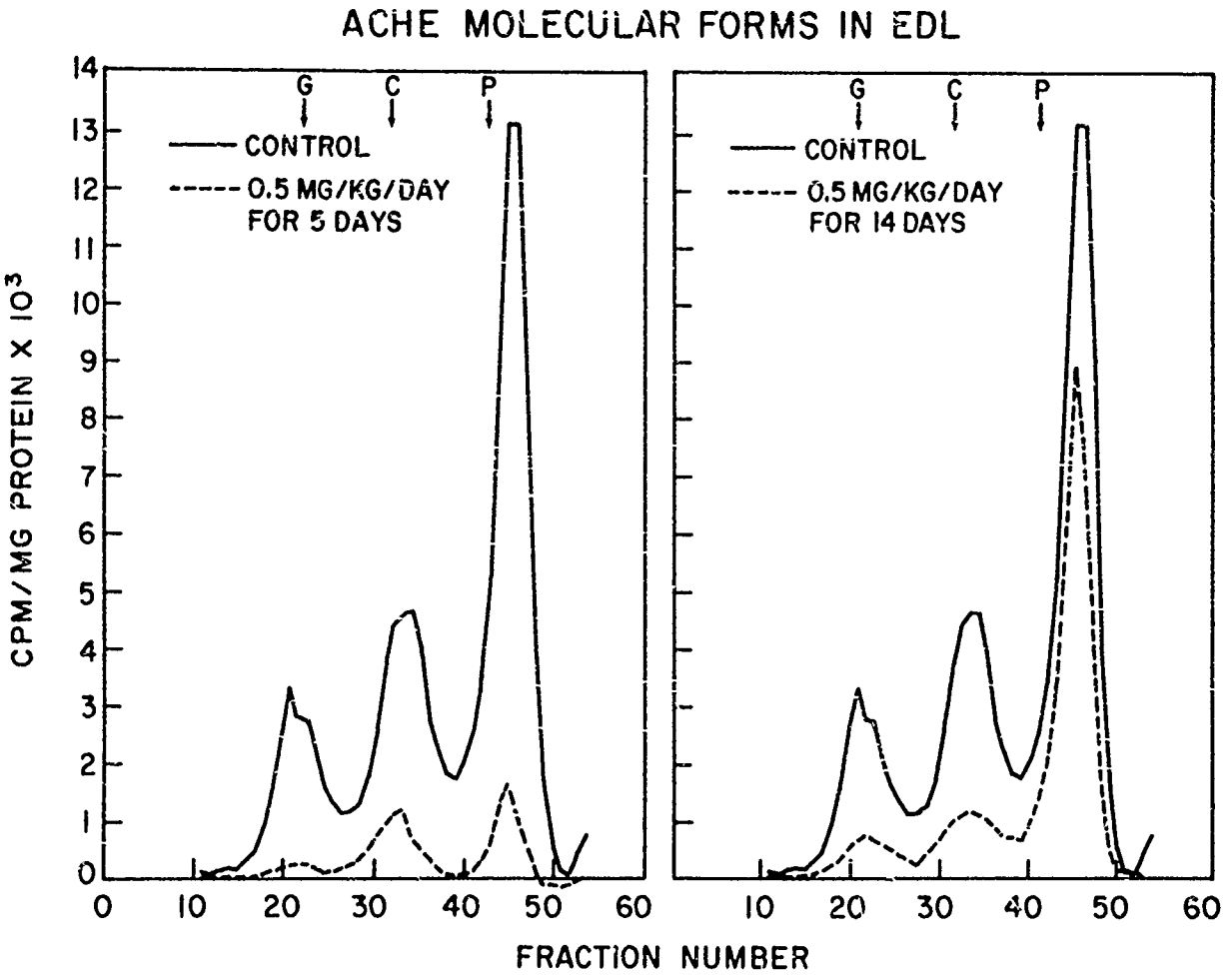

Fig. 2. Effects of DFP administered in repeated doses for 5 days (left) and 14 days (right) on acetylcholinesterase (AChE) molecular forms in EDL muscle. For details see Fig. 1.

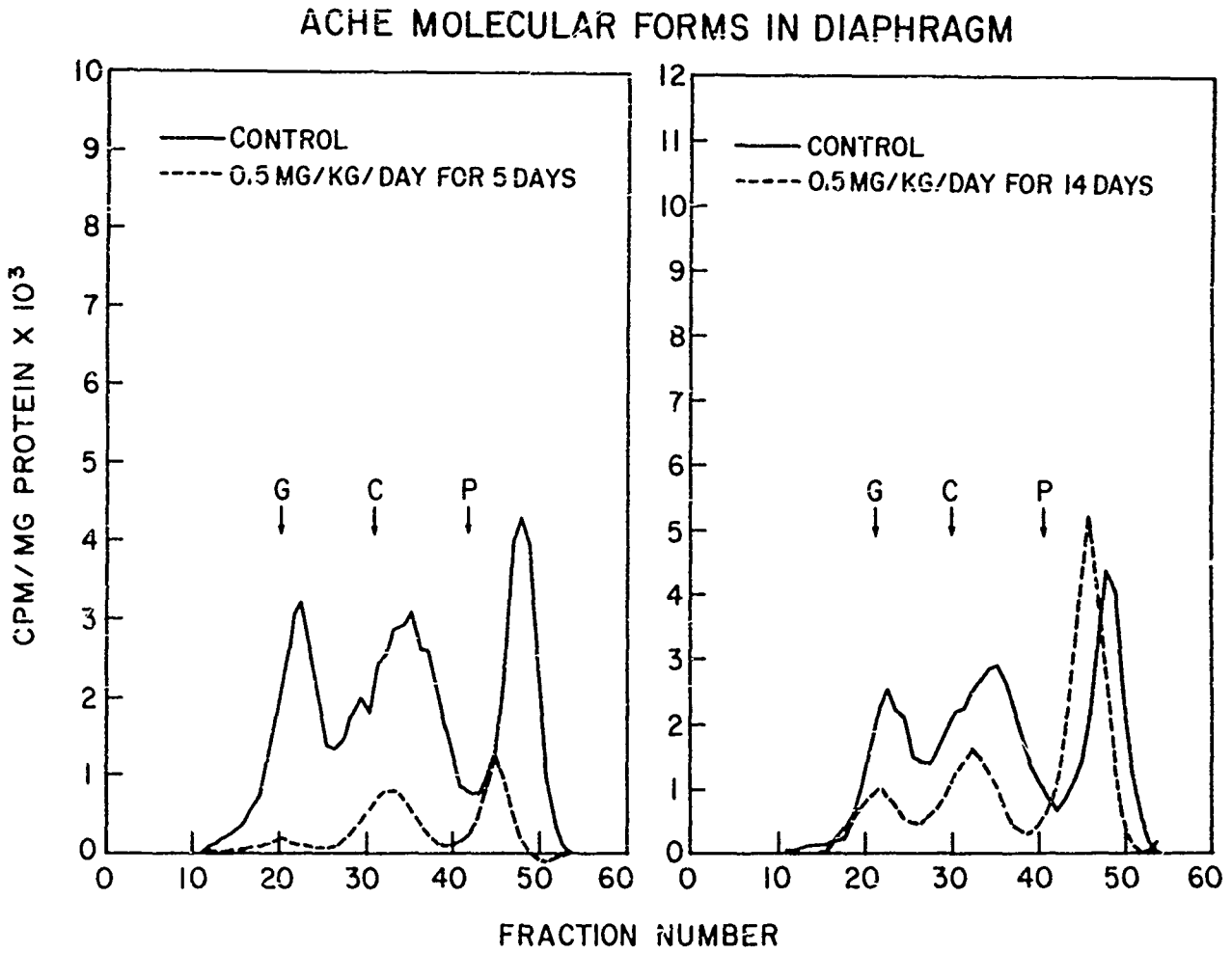

Fig. 3. Effects of DFP administered in repeated doses for 5 days (left) and 14 days (right) on acetylcholinesterase (AChE) molecular forms in diaphragm muscle. For details see Fig. 1.

($p < 0.01$) toward control as compared with the values of day 5. Detailed examination of various molecular forms of AChE activity in skeletal muscles further confirm the above finding of recovery in total AChE activity (Figures 1-3). Maximal recovery is always seen in the activity of the 4S form, which appears first. An exception is the soleus muscle, where all forms have recovered equally well. A significant ($p < 0.05$) recovery was also noticed in BuChE activity of diaphragm and soleus. The activity of AChE and BuChE in brain never recovered from the level observed on day 5.

The activity of ChAT showed significant ($p < 0.01$) elevation in all the muscles tested (Table 3) following repeated DFP administration in both subchronic regimens (day 5 and 14).

Repeated injections of 0.5 mg DFP/kg caused a significant ($p < 0.05$) decrease of nAChR binding sites in diaphragm muscle (Table 4), however, there was no change in the affinity constant.

Neither of the BuChE inhibitors produced a significant ($p > 0.01$) effect on AChE activity in skeletal muscles or in the selected brain regions (Table 5). Both these drugs, however, caused marked inhibition of BuChE in all three skeletal muscles (> 55) and in the brain regions studied ($> 60\%$). Five days of combined treatment with BuChE inhibitors and DFP caused a significantly greater inhibition of AChE in all skeletal muscles ($p < 0.05$) and brain regions ($p < 0.01$) when compared with the effects of DFP alone (Table 6). Similar results were found for BuChE activity in brain, but not in muscles (Table 7).

A single dose of DFP (0.5 mg/kg) reduced aliesterase activity to 20% of control while repeated administrations for five days lowered the activity even more. Further exposure for 14 days resulted in a significant ($p < 0.01$) recovery of aliesterase activity (38%). Single dose treatment of either iso-OMPA or mipafox within 30 min of administration caused significant ($p < 0.01$) inhibition of aliesterase (63% and 84%, respectively). The activity of aliesterase was more than 95% inactivated when rats were treated with a combination of either

TABLE 3. EFFECT OF DFP ADMINISTERED SUBCUTANEOUSLY IN SINGLE AND REPEATED DOSES ON CHOLINE ACETYLTRANSFERASE ACTIVITY IN RAT HEMI-DIAPHRAGM, SLOW SOLEUS AND FAST EXTENSOR DIGITORUM LONGUS (EDL) MUSCLE

Treatment	Dosage	Diaphragm	Soleus	EDL
Control	---	12.51±0.15	4.25±0.08	5.16±0.24
DFP	1.5 mg/kg	13.56±0.21 (+8)	4.66±0.11 (+10)	6.38±0.23 (+24)
DFP	0.5 mg/kg/day for 5 days	17.29±0.53[a] (+38)	6.72±0.30[a] (+58)	7.45±0.36[a] (+44)
DFP	0.5 mg/kg/day for 14 days	17.07±0.51[a] (+36)	6.43±0.11[a] (+51)	6.73±0.28[a] (+30)

Values are mean ± SEM of choline acetyltransferase activity (nmole ACh formed/mg protein/h) obtained from 5-10 animals, and numbers in parentheses represent percent change compared to control.

[a] $p < 0.01$ when compared with values of controls.

TABLE 4. EFFECT OF DFP ADMINISTERED SUBCUTANEOUSLY IN SINGLE AND REPEATED DOSES ON NICOTINIC ACETYLCHOLINE RECEPTORS BINDING BY ^{3}H-ACETYLCHOLINE IN RAT DIAPHRAGM

		^{3}H-Acetylcholine Binding	
Treatment	Dosage	KD	Bmax
Control	---	188.48±25.82	112.28±8.13
DFP	1.5 mg/kg	167.26±17.79 (-11)	80.98±7.11 (-28)
DFP	0.5 mg/kg/day for 5 days	144.09±22.60 (-24)	46.73±2.29[a] (-58)
DFP	0.5 mg/kg/day for 14 days	173.29±29.95 (-8)	49.48±2.43[a] (-56)

Values of KD (PM) and Bmax (fmol/mg protein) were determined from Scatchard analysis of the binding data. Each value is the mean ± SEM obtained from 4-5 animals and numbers in parentheses represent percent change compared to control.

[a] $p < 0.05$ when compared with the values of controls.

TABLE 5. EFFECT OF DAILY ADMINISTRATION OF ISO-OMPA (3 MG/KG, SC/DAY) AND MIPAFOX (0.05 MG/KG, SC/DAY) FOR 5 DAYS ON ACETYLCHOLINESTERASE (ACHE) AND BUTYRYLCHOLINESTERASE (BUCHE) ACTIVITIES IN BRAIN AND SKELETAL MUSCLES OF RAT

	AChE Activity			BuChE Activity		
	Control	iso-OMPA	Mipafox	Control	iso-OMPA	Mipafox
Muscles						
Diaphragm	78.3±1.6 (100)	75.1±2.4 (96)	70.7±1.2 (90)	10.2±0.2 (100)	0.4±0.2[a] (4)	4.2±0.4[a] (41)
Soleus	60.1±1.2 (100)	54.8±2.6 (91)	58.6±1.3 (98)	10.3±0.3 (100)	0.4±0.1[a] (4)	3.9±0.2[a] (38)
EDL	105.8±1.0 (100)	96.4±2.2 (91)	100.9±1.4 (95)	9.7±0.8 (100)	1.2±0.7[a] (12)	4.3±0.4[a] (44)
Brain Regions						
Cortex	271.2±4.0 (100)	222.7±2.3 (82)	266.0±6.3 (98)	18.2±1.1 (100)	5.7±0.4[a] (31)	7.2±0.6[a] (40)
Striatum	1610.4±31.6 (100)	1621.4±26.6 (101)	1575.7±23.9 (98)	20.3±0.5 (100)	5.9±0.4[a] (29)	7.0±0.4[a] (35)
Brain Stem	579.2±11.0 (100)	482.5±12.2 (83)	525.9±11.4 (91)	36.3±1.0 (100)	9.6±0.5[a] (26)	13.3±0.8[a] (36)
Hippocampus	335.8±8.8 (100)	271.9±8.4 (81)	330.9±14.6 (99)	16.4±0.4 (100)	6.9±0.5[a] (42)	6.2±0.3[a] (38)

Values are mean ± SEM of AChE activity (umole acetylthiocholine iodide/g/h) and BuChE activity (umole butyrylthiocholine iodide/g/h) from 5-12 rats. Values in parentheses indicate remaining enzyme activity and expressed as % of control (100%).

[a] $p < 0.01$ when compared with the values of control.

of BuChE inhibitors and DFP for 5 consecutive days. No recovery of aliesterase activity was seen with the drug combinations, and all rats died after the fifth or sixth injection.

DISCUSSION

The present experiments revealed two mechanisms that enabled the rats to adapt to prolonged administration of DFP. (1) recovery of AChE activity despite the continuous presence of DFP in a concentration which initially caused a significant reduction in enzyme activity, and (2) a decrease in the density of the nicotinic receptor sites at the postsynaptic membrane.

The first mechanism is probably due to stimulation of de novo synthesis of AChE, a phenomenon observed in muscle but not in brain. The reason for this difference between these two tissues is not clear; however, preliminary experiments showed a fast recovery of protein synthesis in muscle but not in brain following a similar DFP treatment (unpublished observation). The second mechanism, i.e. reduced receptor sites and their desensitization, appears to be more general since behavioral adaptation involving CNS activity has been reported previously (Russell et al., 1975) despite the lack of recovery of AChE activity. The increased level of ChAT activity observed in our experiments is in agreement with this down regulation of receptor density.

The potentiation of DFP toxicity and the loss of tolerance development in rats pretreated with iso-OMPA or mipafox is probably caused by the reduction of non-AChE binding sites otherwise available for DFP. This would consequently leave more of the DFP to interact with the AChE. More recent studies of Hoskin (1985) suggests that iso-OMPA and mipafox also inhibit DFPase, an enzyme hydrolyzing DFP. Thus, the greater toxicity of DFP in BuChE inhibitors pretreated rats is possibly caused by reduction of non-AChE (unspecific) binding sites and the inhibition of an enzyme that hydrolyzes DFP.

It is concluded from the present experiments that tolerance to DFP toxicity is a result of a number of factor(s) such as:

TABLE 6. ACETYLCHOLINESTERASE (ACHE) ACTIVITY IN BRAIN AND SKELETAL MUSCLES AFTER FIVE DAY TREATMENT WITH DFP (0.5 MG/KG, SC/DAY) AND DFP IN COMBINATION WITH ISO-OMPA (3 MG/KG, SC/DAY) OR MIPAFOX (0.05 MG/KG, SC/DAY)

	AChE Activity			
	Control	DFP	iso-OMPA + DFP	Mipafox + DFP
Muscles				
Diaphragm	78.3+1.6 (100)	16.9+3.0[a] (22)	1.4+0.6[a,b] (2)	6.0+0.9[a,b] (8)
Soleus	60.1+1.2 (100)	11.3+1.8[a] (19)	0.9+0.6[a,b] (1)	3.4+1.0[a,b] (5)
EDL	105.8+1.0 (100)	20.8+2.5[a] (20)	5.6+1.1[a,b] (5)	4.3+0.4[a,b] (4)
Brain Regions				
Cortex	271.2+4.0 (100)	27.8+5.1[a] (10)	7.0+0.8[a,c] (3)	8.7+1.4[a,c] (3)
Striatum	1610.4+31.6 (100)	142.1+4.3[a] (9)	65.7+3.3[a,c] (4)	59.2+2.7[a,c] (4)
Brain Stem	579.2+11.0 (100)	111.6+1.4[a] (19)	75.8+5.5[a,b] (13)	60.5+5.0[a,c] (10)
Hippocampus	335.8+8.8 (100)	53.4+1.4[a] (16)	32.6+1.6[a,c] (10)	29.5+1.5[a,c] (9)

Values are the mean + SEM of AChE activity (umol acetylthiocholine iodide/g/h) from 5-12 animals. Values in parentheses indicate remaining AChE activity and expressed as % of control (100%).

[a] $p < 0.01$ when compared with the values of controls.
[b] $p < 0.05$; [c] $p < 0.01$ when values of iso-OMPA or mipafox in combination with DFP compared with the values of DFP alone.

TABLE 7. BUTYRYLCHOLINESTERASE (BUCHE) ACTIVITY IN BRAIN AND SKELETAL MUSCLES AFTER FIVE DAY TREATMENT WITH DFP (0.5 MG/KG, SC/DAY) AND DFP IN COMBINATION WITH ISO-OMPA (3 MG/KG, SC/DAY) OR MIPAFOX (0.05 MG/KG, SC/DAY)

	BuChE Activity			
	Control	DFP	iso-OMPA + DFP	Mipafox + DFP
Muscles				
Diaphragm	10.2+0.2 (100)	0.8+0.3[a] (8)	0.9+0.4[a] (9)	0.5+0.2[a] (5)
Soleus	10.3+0.3 (100)	2.4+1.2[a] (23)	2.5+0.6[a] (24)	0.6+0.3[a] (6)
EDL	9.7+0.8 (100)	4.7+1.2[a] (48)	0.6+0.2[a] (6)	0.3+0.1[a] (3)
Brain Regions				
Cortex	18.2+1.1 (100)	6.8+0.6[a] (37)	2.7+0.5[a,b] (15)	3.2+0.3[a,b] (18)
Striatum	20.3+0.5 (100)	6.6+0.3[a] (33)	2.2+0.5[a,b] (11)	3.3+0.9[a,b] (16)
Brain Stem	36.3+1.0 (100)	10.0+0.5[a] (28)	4.6+0.8[a,b] (13)	3.3+0.3[a,c] (9)
Hippocampus	16.4+0.4 (100)	7.1+0.3[a] (43)	1.2+0.6[a,c] (7)	1.9+0.6[a,c] (12)

Values are mean + SEM of BuChE activity (umol butyrylthiocholine iodide/g/h) from 5-12 animals. Values in parentheses indicate remaining BuChE activity and expressed as % of control (100%).

[a] $p < 0.01$ when compared with the values of controls.
[b] $p < 0.05$; [c] $p < 0.01$ when values of iso-OMPA or mipafox in combination with DFP compared with the values of DFP alone.

TABLE 8. EFFECT OF DFP ADMINISTRATION ALONE OR FOLLOWING ISO-OMPA OR MIPAFOX PRETREATMENT ON LIVER ALIESTERASE ACTIVITY

Treatment	Sacrifice Time After Last Treatment (h)	Aliesterase Activity	Percent Activity
Single Dose Treatment			
Control (no treatment)	---	274.1 ± 3.9	100
iso-OMPA, 3 mg/kg	24	131.4 ± 3.1[a]	48
Mipafox, 0.05 mg/kg	24	69.3 ± 4.0[a]	25
DFP, 0.5 mg/kg	24	53.3 ± 4.4[a]	19
Repeated Dose Treatment			
DFP, 0.5 mg/kg/day for 5 days	24	22.6 ± 3.5[a]	8
DFP, 0.5 mg/kg/day for 14 days	24	105.0 ± 7.2[a,b]	38
iso-OMPA, 3 mg/kg for 5 days	24	28.6 ± 6.7[a]	10
Mipafox, 0.05 mg/kg for 5 days	24	18.7 ± 2.0[a]	7
iso-OMPA, 3 mg/kg/day + DFP, 0.5 mg/kg/day for 5 days	24	15.0 ± 1.4[a]	5
Mipafox, 0.05 mg/kg/day + DFP, 0.5 mg/kg/day for 5 days	24	15.7 ± 5.1[a]	5

Values are mean ± SEM of aliesterase activity (nmole tributyrin/mg protein/min) from 5-10 rats.

[a] $p < 0.01$ when compared with the values of controls.
[b] $p < 0.01$ when values of day 14 compared with the values of day 5.

(a) de novo synthesis of AChE in muscles, (b) decreased nicotinic acetylcholine receptor sites, and (c) increased non-specific binding sites.

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INTERACTIONS OF ORGANOPHOSPHATE NERVE AGENTS WITH THE NICOTINIC ACETYLCHOLINE RECEPTOR

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ABSTRACT

ORGANOPHOSPHATE (OP) ANTICHOLINESTERASE AGENTS ARE POTENT INHIBITORS OF ACETYLCHOLINE (ACh) ESTERASE, BUT THEY ALSO INTERACT WITH THE NICOTINIC ACh-RECEPTOR, THOUGH WITH LOWER POTENCY. THE EFFECTS OF SOMAN, VX, DFP AND OTHER OP ANTICHOLINESTERASES ON THE BINDING OF RADIOLABELED LIGANDS TO ACh-RECEPTOR OF *TORPEDO* ELECTRIC ORGAN WERE STUDIED. α-BUNGAROTOXIN (α-BGT) IS A SPECIFIC COMPETITIVE INHIBITOR OF NICOTINIC ACh-RECEPTORS OF *TORPEDO* ELECTRIC ORGAN AND VERTEBRATE SKELETAL MUSCLES, WHILE PERHYDROHISTRIONICOTOXIN (H_{12}-HTX) AND PHENCYCLIDINE (PCP) ARE NONCOMPETITIVE INHIBITORS. THUS [^{125}I]α-BGT IS USED TO LABEL THE RECEPTOR'S RECOGNITION SITES, AND [^{3}H]H_{12}-HTX OR [^{3}H]PCP IS USED TO LABEL THE ALLOSTERIC CHANNEL SITES. BINDING OF [^{3}H]H_{12}-HTX OR [^{3}H]PCP IS VERY SLOW TO RESTING RECEPTORS, BUT BECOMES FAST IN PRESENCE OF RECEPTOR AGONISTS WHERE RECEPTORS EXIST IN ACTIVATED OR DESENSITIZED STATES.

AT LOW CONCENTRATIONS ALL OP'S TESTED INHIBITED ACh-ESTERASE IN *TORPEDO* MEMBRANES AND POTENTIATED THE STIMULATION BY ACh OF [^{3}H]H_{12}-HTX OR [^{3}H]PCP BINDING. HOWEVER, AT HIGHER CONCENTRATION, THEY INHIBITED THE EFFECTS OF ACh NONCOMPETITIVELY. VX WAS THE MOST POTENT INHIBITOR OF [^{3}H]H_{12}-HTX BINDING TO THE CHANNEL SITE, WHETHER IN CLOSED OR ACTIVATED RECEPTOR CONFORMATION, BUT MUCH MORE TO THE LATTER CONFORMATION. THE K_I VALUE FOR INHIBITION OF [^{3}H]H_{12}-HTX BINDING TO THE CHANNEL SITE BY SOMAN WAS 30 μM. DFP, PARAOXON AND ECHOTHIOPHATE INCREASED THE APPARENT RATE OF [^{3}H]PCP BINDING TO *TORPEDO* ACh-RECEPTORS, AND THESE EFFECTS WERE BLOCKED BY PRETREATMENT OF THE MEMBRANES WITH THE COMPETITIVE ANTAGONIST *NAJA* α-NEUROTOXIN. HOWEVER, ONLY ECHOTHIOPHATE DISPLACED [^{125}I]α-BGT BINDING. THESE EFFECTS OF OP'S ON [^{3}H]PCP BINDING WERE TIME DEPENDENT. THUS, OP COMPOUNDS, INCLUDING NERVE AGENTS, HAVE MULTIPLE SITES OF ACTION AT CHOLINERGIC SYNAPSES AND HAVE DIFFERENT ACTIONS ON THE NICOTINIC ACh-RECEPTOR.

(THIS WORK WAS SUPPORTED IN PART BY THE US ARMY ARMAMENT, MUNITIONS AND CHEMICAL COMMAND UNDER CONTRACT DAAK11-84-K-0006 AND DAMD-17-81-C-1279.)

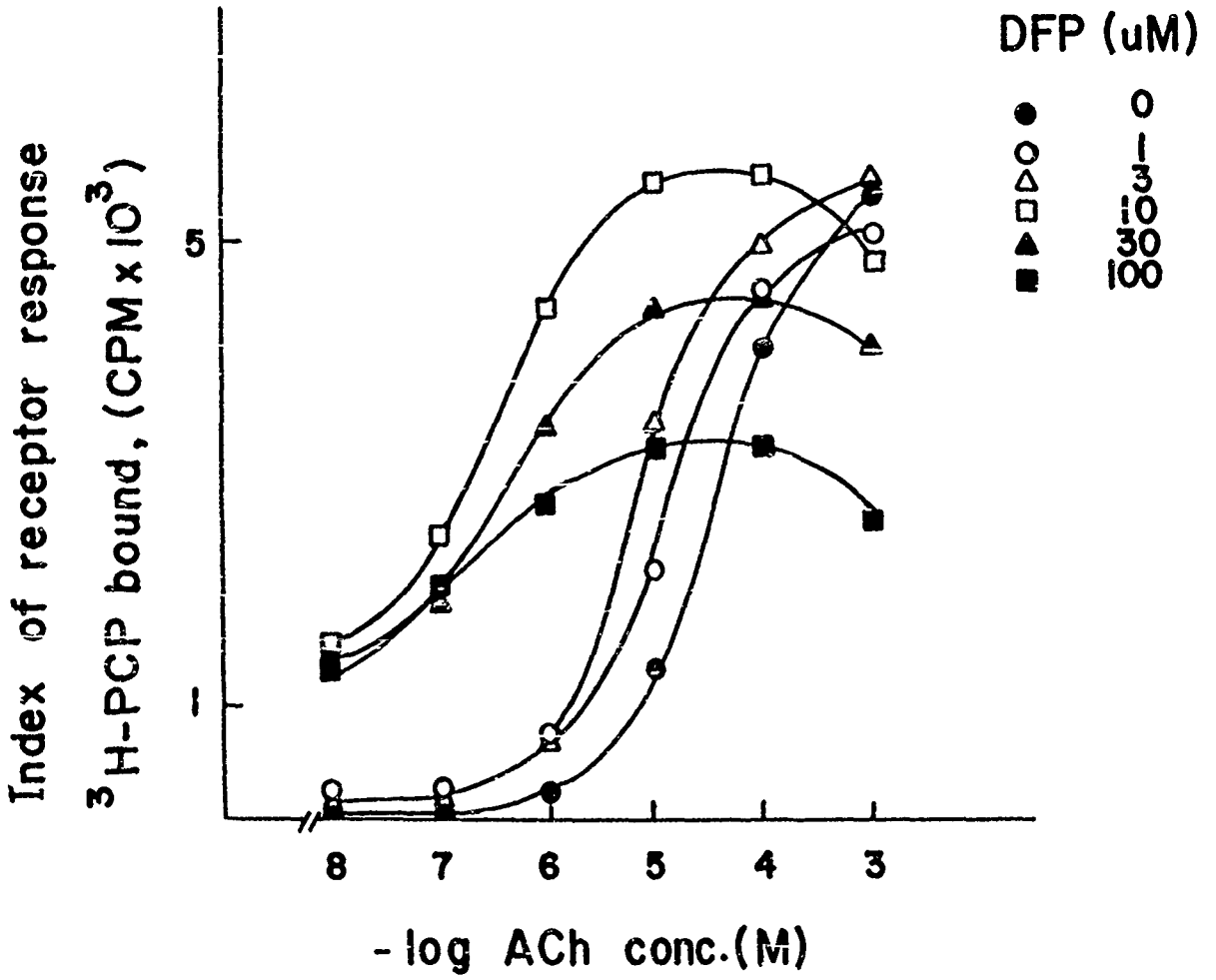
EXPERIMENTAL METHODS

A membrane preparation from Torpedo nobiliana electric organs enriched in ACh receptors (≈350 pmole/mg protein) was obtained by differential centrifugation (see Sherby, S.M., Eldefrawi, A.T., Albuquerque, E.X., and Eldefrawi, M.E. (1984) Mol. Pharmacol. 27, 343) and used to study the effect of several organophosphate (OP) anticholinesterases on binding of radioactive ligands to the ACh receptor.

^{125}I-α-Bungarotoxin (BGT) was used to label the receptor site and to identify OPs capable of binding to the receptor site and displacing ^{125}I-α-BGT binding. Specific (i.e., Naja α-neurotoxin sensitive) binding of ^{125}I-α-BGT was measured by an ion exchange minicolumn assay described previously (Kohanski, R.A., Andrews, J.P., Wins, P., Eldefrawi, M.E., and Hess, G.P. (1977) Analyt. Biochem. 80, 531).

^{3}H-Perhydrohistrionicotoxin (H_{12}-HTX) and ^{3}H-phencyclidine (PCP) were used to label sites on the ionic channel, also known as noncompetitive blockers, sites using a filtration assay as described (Aronstam, R.S., Eldefrawi, A.T., Pessah, I.N., Daly, J.W., Albuquerque, E.X., and Eldefrawi, M.E. (1981) J. Biol. Chem. 256, 2843; Eldefrawi, M.E., Eldefrawi, A.T., Aronstam, R.S., Maleque, M.A., Warnick, J.W., and Albuquerque, E.X. (1980) Proc. Natl. Acad. Sci. USA 77, 7458).

Acetylcholinesterase activity in the membrane preparation was determined by the Ellman spectrophotometric method (Ellman, G.L., Courtney, K.D., Andres, V., Jr., and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88).



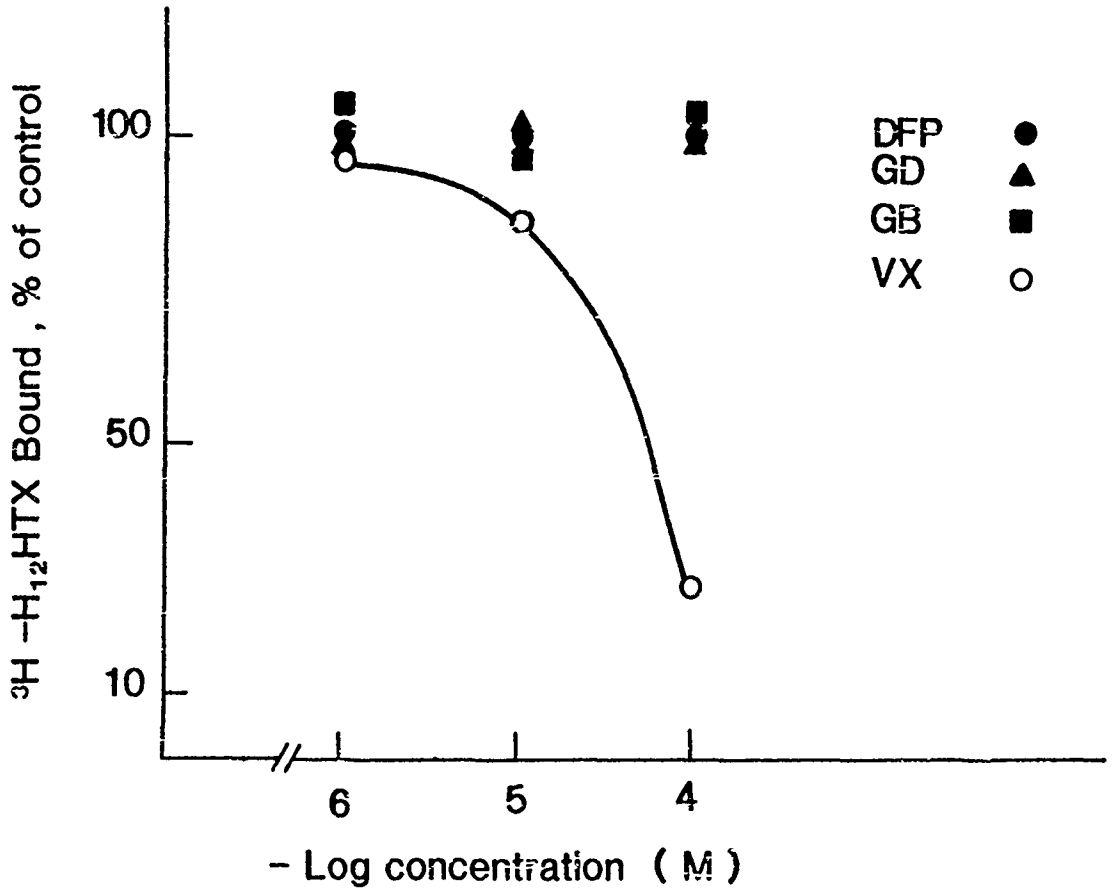
EFFECT OF DFP ON THE ACH-INDUCED BINDING OF ^{3}H-PCP TO TORPEDO ACH RECEPTORS.

ACH INCREASES THE INITIAL RATE OF ^{3}H-PCP BINDING (30 SEC INCUBATION) IN A DOSE-DEPENDENT MANNER. THUS, ^{3}H-PCP BINDING REFLECTS RECEPTOR RESPONSE.

DFP AT LOW CONCENTRATIONS (1-10 μM) SHIFTS THE DOSE-RESPONSE FUNCTION TO THE LEFT BECAUSE INHIBITION OF ACH-ESTERASE IN THE TISSUE PROTECTS ACH FROM HYDROLYSIS AND POTENTIATES ITS EFFECT.

AT HIGHER CONCENTRATIONS (>10 μM) DFP REDUCES THE MAXIMAL EFFECT OF ACH, SUGGESTING NONCOMPETITIVE INHIBITION.

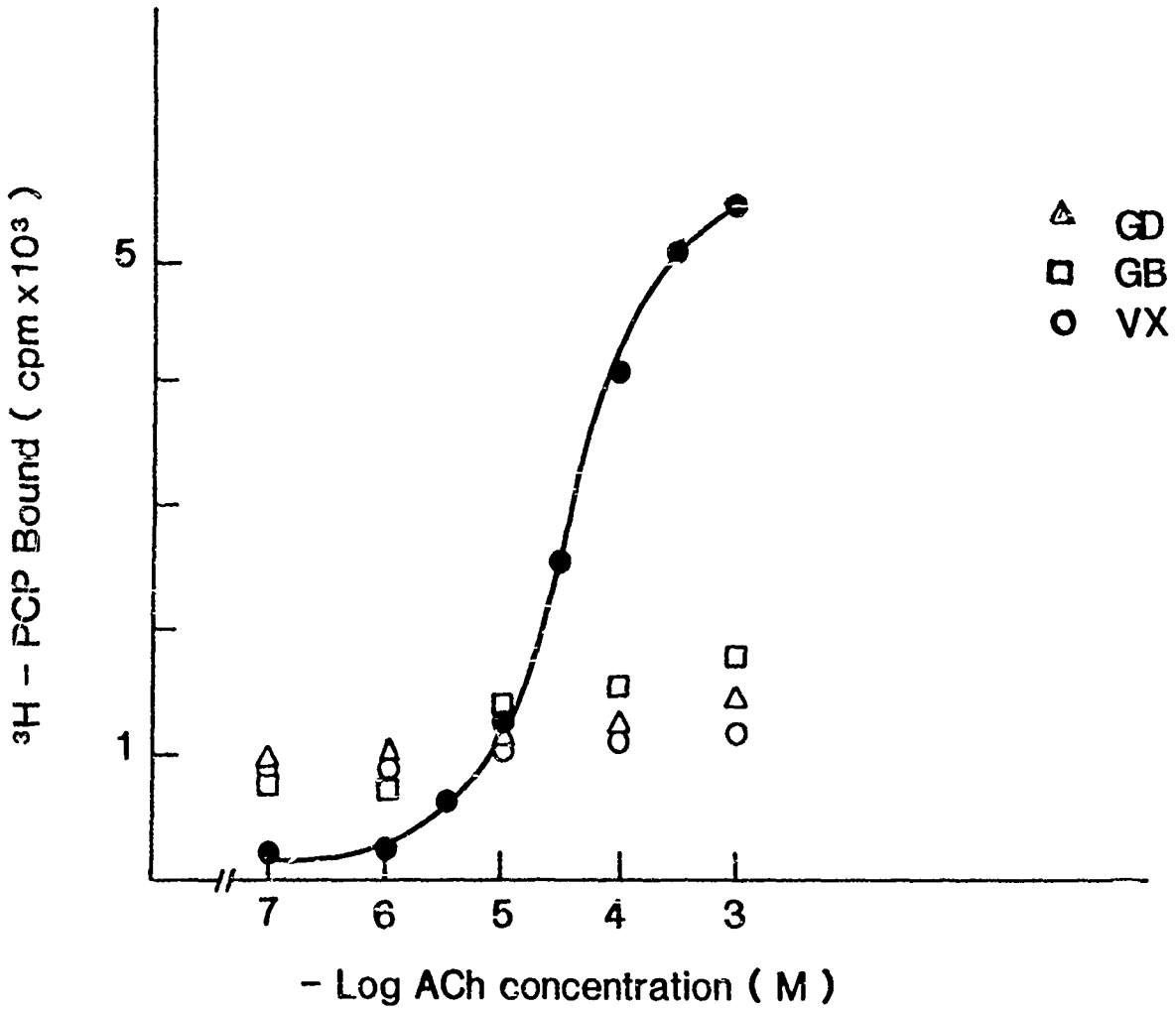
TO STUDY EFFECTS OF DFP, TORPEDO MEMBRANES WERE TREATED FOR 30 MIN PRIOR TO ASSAY. DFP AT 1, 3 AND 10 μM GAVE 44%, 73% AND 100% INHIBITION OF ACH-ESTERASE, RESPECTIVELY.



THE EFFECT OF DFP, GD, GB & VX ON THE EQUILIBRIUM BINDING (120 MIN INCUBATION) OF ^3H-H_{12}-HTX TO RESTING TORPEDO ACH RECEPTORS IN ABSENCE OF RECEPTOR ACTIVATION (I.E., NO ACH ADDED).

ONLY VX DISPLACED ^3H-H_{12} HTX EQUILIBRIUM BINDING TO RESTING ACH RECEPTORS. THESE RESULTS SUGGEST THAT VX IS A CLOSED CHANNEL BLOCKER. IT IS POSSIBLE THE VX IS ALSO AN OPEN CHANNEL BLOCKER AS SHOWN IN FIG. 3.

IF REDUCTION OF ^3H-PCP BINDING TO ACTIVATED ACH RECEPTORS BY GD AND GB IS DUE TO CHANNEL BLOCKADE, THEN THESE TWO AGENTS ARE OPEN CHANNEL BLOCKERS ONLY, WHEREAS VX CAN BLOCK BOTH OPEN AND CLOSED RECEPTOR CHANNELS.



EFFECT OF THE OP NERVE AGENTS GD, GB & VX (1 uM) ON THE ACH-INDUCED ^{3}H-PCP BINDING TO TORPEDO ACH RECEPTORS.

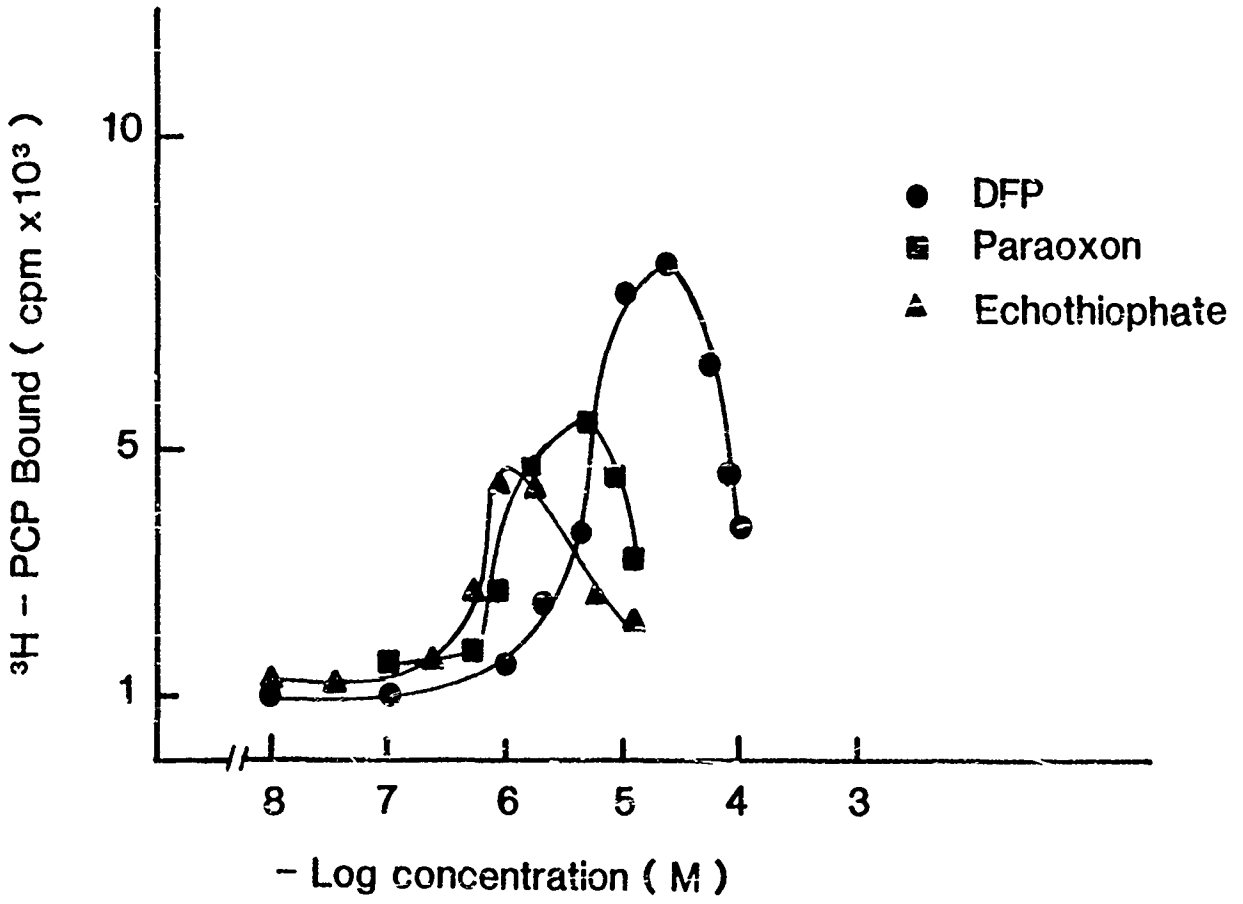
ORGANOPHOSPHORUS NERVE AGENTS (E.G., GD, GB AND VX) AT 1 uM GAVE TOTAL INHIBITION OF ACH-ESTERASE ACTIVITY IN THE TISSUE.

LOWER CONCENTRATIONS (E.G., 100 nM) GAVE PARTIAL ACH-ESTERASE INHIBITION AND SHIFTED THE ACH DOSE-RESPONSE FUNCTION TO THE LEFT.

AT 1 uM THESE AGENTS WERE POTENT INHIBITORS OF ACH-INDUCED ^{3}H-PCP BINDING. AGAIN REDUCTION OF THE MAXIMAL RESPONSE SUGGESTS NONCOMPETITIVE INHIBITION.

THE THREE AGENTS AT 1 uM, 30 MIN INCUBATION, GAVE 95-100% INHIBITION OF ACH-ESTERASE ACTIVITY.

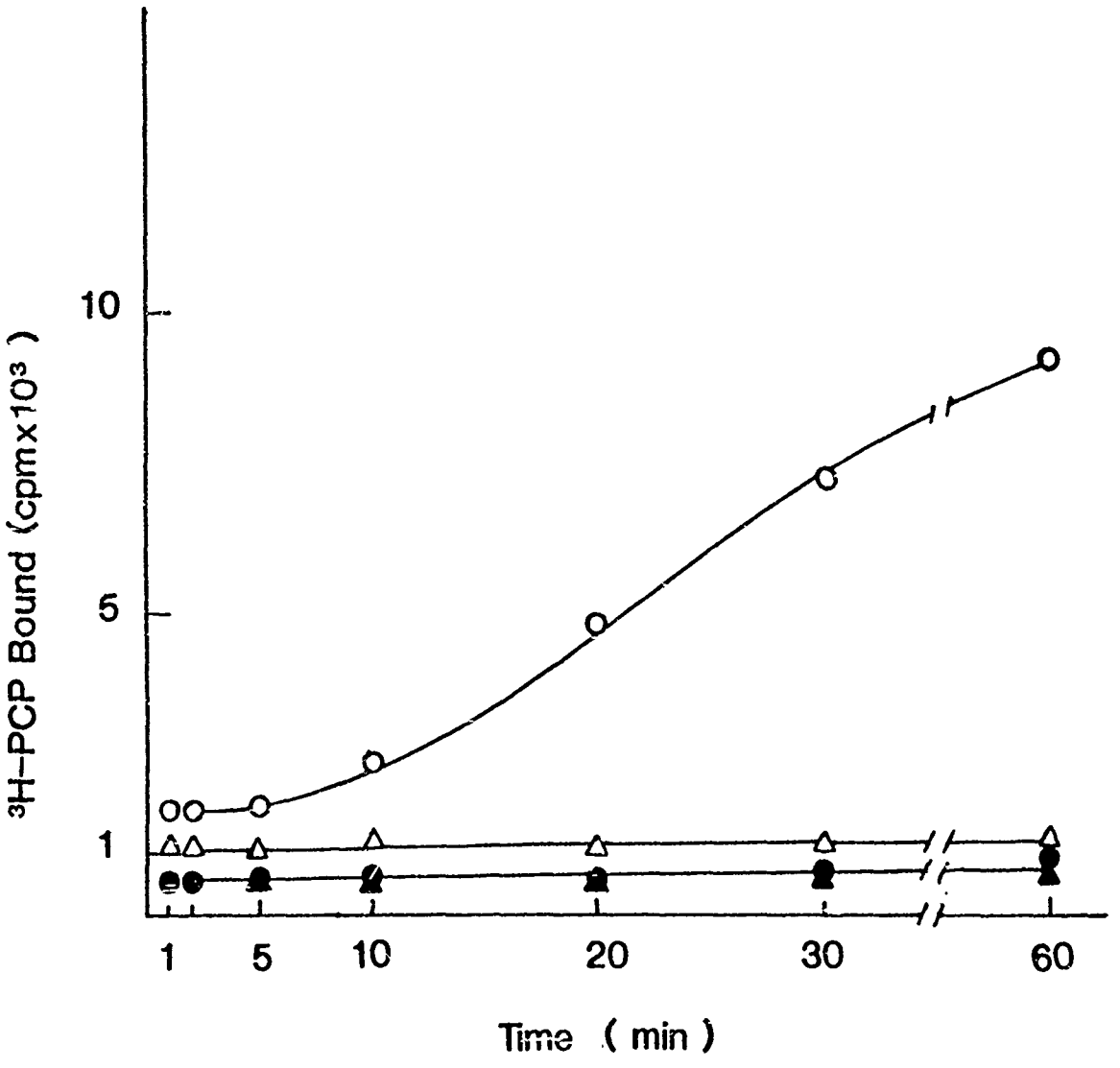
THE SIGNIFICANT REDUCTION IN ^{3}H-PCP BINDING AT HIGHER ACH CONCENTRATIONS SUGGESTS THAT THE NERVE AGENTS MAY BE ACTING AS OPEN CHANNEL BLOCKERS (I.E., BLOCKING OPEN CHANNELS ONLY).



EFFECT OF DFP, PARAOXON AND ECHOTHIOPHATE ON THE INITIAL RATE OF BINDING OF ^{3}H-PCP TO RESTING TORPEDO ACH RECEPTORS. ALL OPS WERE INCUBATED WITH THE TISSUE FOR 60 MIN PRIOR TO ASSAY.

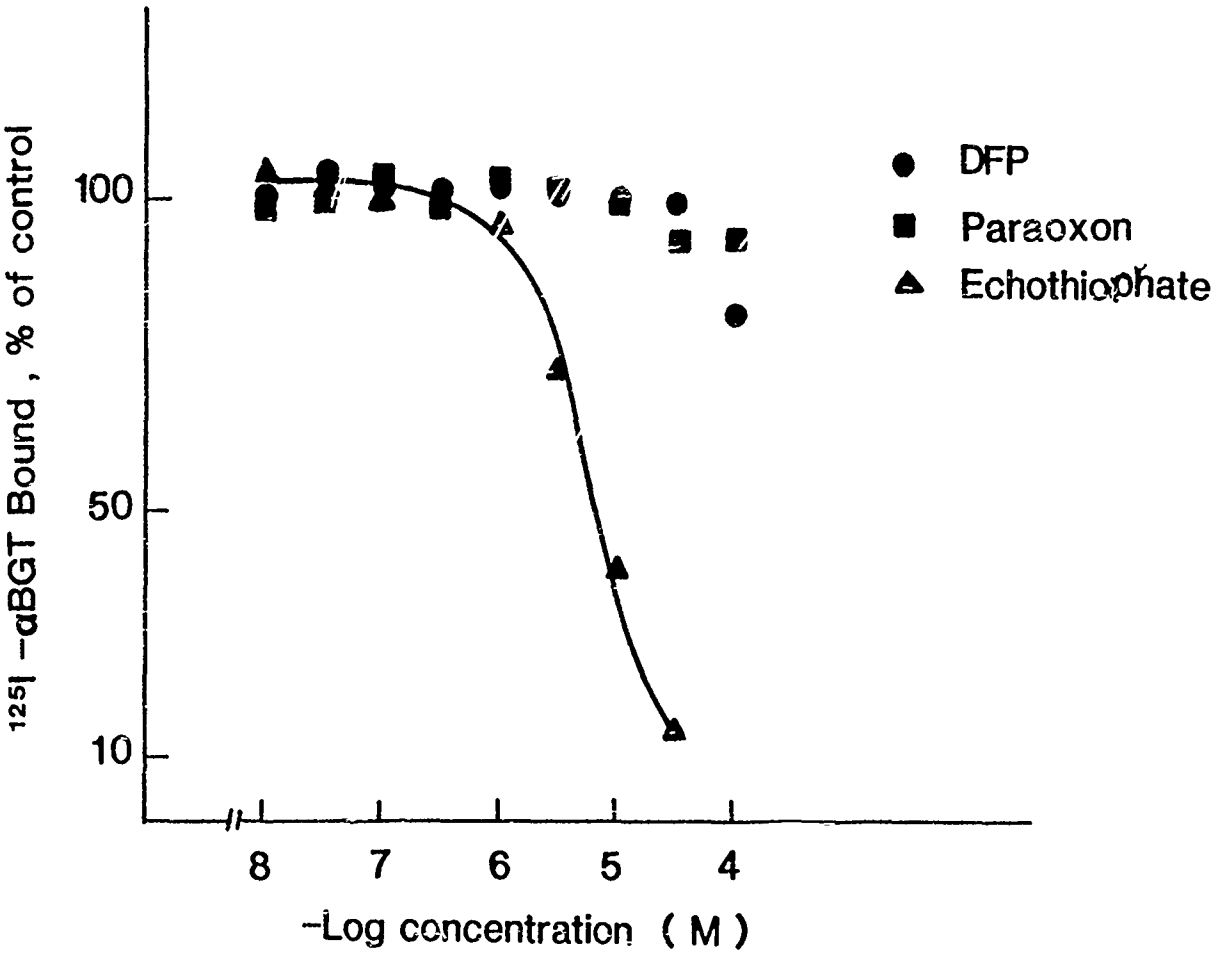
THESE RESULTS DEMONSTRATE THAT MANY ANTICHOLINESTERASES BIND TO THE ACH RECEPTOR IN THE SUBMICROMOLAR RANGE, AND THEIR INTERACTIONS MAY BE DETECTED BY THE EFFECT THEY PRODUCE ON ^{3}H-PCP BINDING.

MOREOVER, IT IS OBVIOUS THAT ALL COMPOUNDS PRODUCE A BIPHASIC EFFECT WHICH MAY REFLECT BINDING OF THE OP TO MORE THAN ONE SITE.



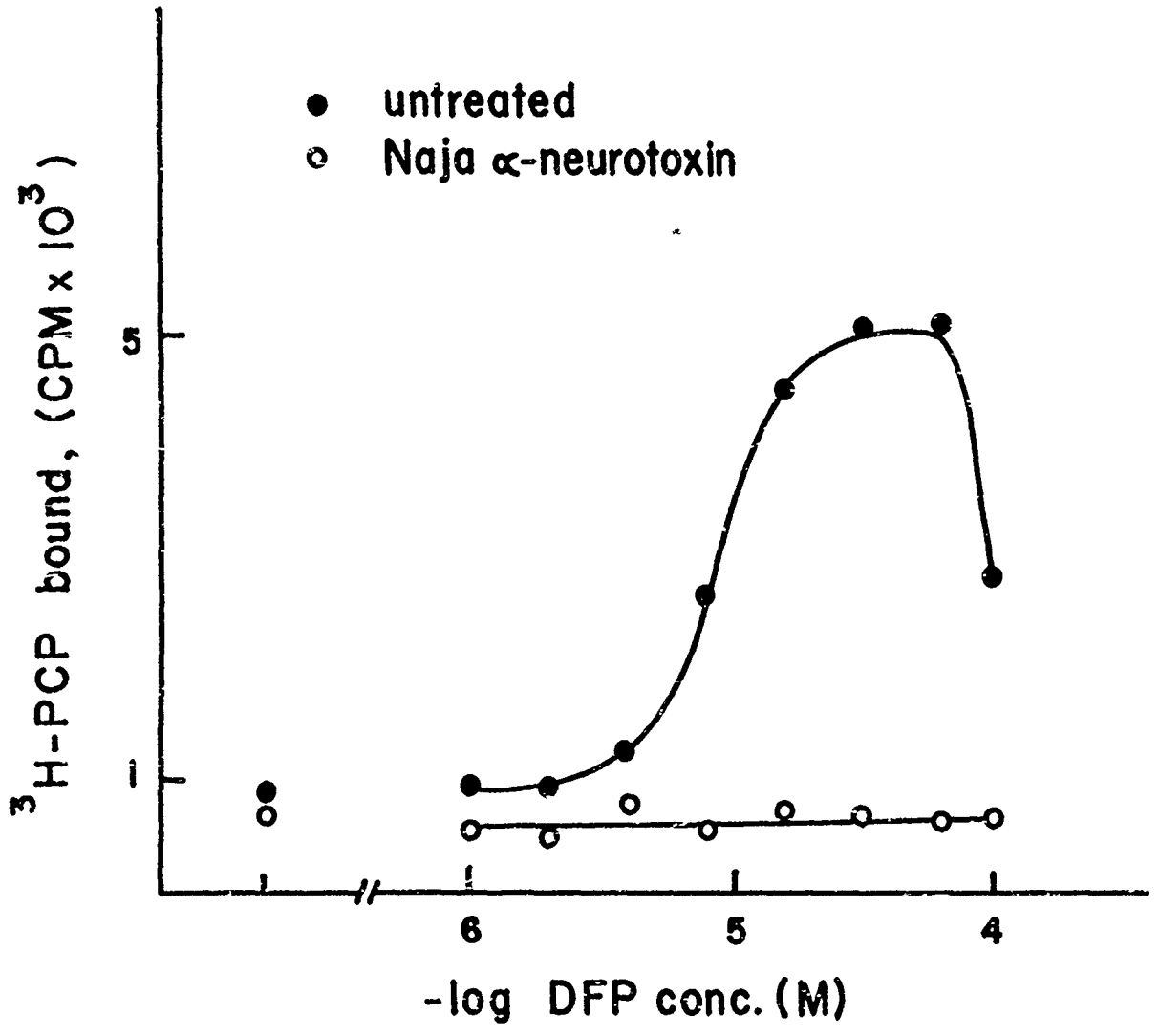
EFFECT OF DFP (10 μM) ON ^{3}H-PCP BINDING TO TORPEDO ACH RECEPTORS AS A FUNCTION OF TIME. NAJA α-NEUROTOXIN-TREATED TISSUE (●,▲), UNTREATED TISSUE (○,△). CONTROL (NO DFP TREATMENT) (△,▲).

DFP (10 μM) HAS A TIME-DEPENDENT ACTION RESULTING IN INCREASED ^{3}H-PCP BINDING, AND THIS ACTION IS BLOCKED WHEN THE RECEPTOR SITES ARE OCCUPIED BY NAJA α-NEUROTOXIN.



EFFECT OF DFP, PARAOXON AND ECHOTHIOPHATE ON THE BINDING OF ^{125}I-α-BGT TO TORPEDO ACH RECEPTORS. TISSUE WAS PREINCUBATED WITH OPS FOR 60 MIN PRIOR TO MIXING WITH ^{125}I-α-BGT (5 nM) FOR 40 SEC.

OTHIOPHATE, WHICH IS A CHOLINE ESTER, BINDS TO THE RECEPTOR SITE AND DISPLACES ^{125}I-α-BGT COMPETITIVELY. DFP AND PARAOXON, HOWEVER, HAVE NO SIGNIFICANT EFFECT UP TO 100 nM.



EFFECT OF DFP ON THE INITIAL RATE OF BINDING OF ^{3}H-PCP TO RESTING TORPEDO ACH RECEPTORS; UNTREATED (•) AND TREATED WITH 10 μM NAJA α-NEUROTOXIN (o). DFP WAS PREINCUBATED WITH THE TISSUE FOR 60 MIN PRIOR TO ADDITION OF ^{3}H-PCP, MIXING AND FILTRATION AFTER 30 SEC.

IT IS OBVIOUS THAT DFP IS BINDING TO THE ACH RECEPTOR PROTEIN AND ALLOSTERICALLY MODIFYING THE ^{3}H-PCP BINDING SITE TO INCREASE THE BINDING RATE.

MOREOVER THIS DFP EFFECT IS TOTALLY BLOCKED BY PRIOR OCCUPATION OF THE RECEPTOR SITES BY THE IRREVERSIBLE ANTAGONIST NAJA α-NEUROTOXIN.

CONCLUSIONS

1. OP ANTICHOLINESTERASES BIND TO AND MODULATE THE ACTIVITY OF NICOTINIC ACh RECEPTORS OF TORPEDO ELECTRIC ORGANS.

2. SOME OP ANTICHOLINESTERASES SUCH AS ECHOTHIOPHATE MAY BIND TO THE RECEPTOR SITE AND MAY ACT AS AGONISTS OR ANTAGONISTS.

3. MOST OP ANTICHOLINESTERASES BIND TO ALLOSTERIC SITES ON THE ACh RECEPTOR MOLECULE AND INTERFERE WITH ACh ACTION ON THE RECEPTOR.

4. THESE ALLOSTERIC INTERACTIONS OCCUR IN THE SAME CONCENTRATION RANGE (nM TO μM) AS ANTICHOLINESTERASE ACTION.

5. SOME OP MAY BIND TO THE NONCOMPETITIVE BLOCKER SITES ON THE IONIC CHANNEL PORTION OF THE RECEPTOR MOLECULE AND ACT AS BLOCKERS OF ACTIVATED RECEPTORS (E.G., DFP, GD, GB).

6. THE OP NERVE AGENT VX APPEARS TO INTERACT WITH BOTH ACTIVATED AND RESTING RECEPTORS.

7. THEREFORE THE EFFECT OF OP AGENTS AT NICOTINIC CHOLINERGIC SYNAPSES IS NOT LIMITED TO THEIR ANTICHOLINESTERASE ACTION, BUT SHOULD INCLUDE THEIR ACTION ON THE ACh RECEPTOR.

PROTECTION BY CALCIUM CHANNEL ANTAGONISTS AGAINST ORGANOPHOSPHATE POISONING

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CURRENTLY, THE BEST PROTECTION AGAINST THE TOXIC EFFECTS OF ORGANOPHOSPHATES, SUCH AS DIISOPROPYLFLUOROPHOSPHATE (DFP), IS PRODUCED BY ATROPINE (AP) AND 2-PRALIDOXIME (2-PAM). IN ADDITION TO INHIBITING CHOLINESTERASE, ORGANOPHOSPHATES INCREASE PRESYNAPTIC RELEASE OF ACETYLCHOLINE AT CHOLINERGIC SYNAPSES PERHAPS BY PROMOTING CALCIUM INFLUX. OUR PURPOSE WAS TO EVALUATE THE PROTECTIVE ABILITIES OF CALCIUM CHANNEL BLOCKING AGENTS IN THE PRESENCE OR ABSENCE OF THE STANDARD COMBINED PRETREATMENT (SCP) OF AP AND 2-PAM.

AN EVALUATION OF THE PROTECTIVE EFFECTS OF THESE AGENTS OVER A WIDE DOSE RANGE WAS DONE USING STANDARD LD_{50} STUDIES IN MICE. THE LD_{50} FOR DFP ALONE WAS 6.0 MG/KG. AFTER AP, 1.0 MG/KG, THE LD_{50} OF DFP WAS 7.3 MG/KG*; 2-PAM, 10.0 MG/KG, RAISED THE LD_{50} TO 8.1 MG/KG*, WHILE THE SCP RAISED THE LD_{50} TO 8.7 MG/KG*. THE CALCIUM CHANNEL BLOCKERS PROTECTED AS FOLLOWS: VERAPAMIL (V), 3.0 MG/KG, RAISED THE LD_{50} TO 7.5 MG/KG*; COMBINED WITH THE SCP THE LD_{50} WAS INCREASED TO 9.3 MG/KG*. NIFEDIPINE (NF), 0.05 MG/KG, RAISED THE LD_{50} TO 8.8 MG/KG* AND COMBINED WITH THE SCP, TO 13.6 MG/KG*. NITRENDIPINE, NIMODIPINE, AND DILTIAZEM DID NOT SIGNIFICANTLY INCREASE THE PROTECTION AFFORDED BY THE SCP.

PHENYTOIN (PH), 15.0 MG/KG, RAISED THE LD_{50} TO 6.8 MG/KG* ; AFTER PH PLUS THE SCP, THE LD_{50} WAS INCREASED TO 10.6 MG/KG*. PROTECTION PROVIDED BY PH WAS NOT DUE TO ITS ANTICONVULSANT PROPERTIES PER SE, AS CARBAMAZEPINE, PHENOBARBITAL, AND DIPHENYLBARITURATE WERE WITHOUT EFFECT.

IN ORDER TO DETERMINE WHETHER THESE EFFECTS WERE CENTRAL, PERIPHERAL OR BOTH, WE RECORDED EEG AND NEURALLY EVOKED GASTROCNEMIUS MUSCLE CONTRACTIONS IN CATS ANESTHETIZED WITH ALPHA CHLORALOSE. DFP WAS ADMINISTERED I.V. IN CUMULATIVE DOSES AND THE AMOUNT NEEDED TO INDUCE NEUROMUSCULAR FAILURE (NMF) AND MAXIMUM EEG SEIZURE ACTIVITY (MS) WAS DETERMINED. IN CONTROL ANIMALS, NMF WAS OBSERVED AT 4.2 MG/KG WHILE MS OCCURRED AT 3.6 MG/KG. AP PROVIDED PROTECTION ONLY AGAINST THE CENTRAL EFFECTS WHEREAS PH EXERTED ACTIONS BOTH CENTRALLY AND PERIPHERALLY. MAXIMUM PROTECTION WAS SEEN WITH THE COMBINED PRETREATMENT OF AP AND PH.

THESE STUDIES SHOWED THAT THE TOXIC EFFECTS OF DFP WERE MANIFESTED AT LOWER DOSES CENTRALLY THAN PERIPHERALLY AND THAT PH AS WELL AS AP PROTECT THE CENTRAL SITES. SINCE THE MAJOR CAUSE OF DEATH BY THE ORGANOPHOSPHATES IS RESPIRATORY DEPRESSION, WE INVESTIGATED THE EFFECTS OF DFP ON CENTRALLY MEDIATED RESPIRATORY OUTFLOW AND COMPARED THEM WITH NEUROMUSCULAR EVENTS BY RECORDING INTEGRATED PHRENIC NERVE BURSTING ACTIVITY CONCOMITANT WITH GASTROCNEMIUS MUSCLE CONTRACTIONS. PHRENIC NERVE ACTIVITY CONSISTENTLY CEASED PRIOR TO NMF. CESSATION OF PHRENIC NERVE BURSTS (CPN) OCCURRED IN UNPROTECTED CATS AT 2.3 MG/KG. PRETREATMENT WITH AP ELEVATED THIS DOSE TO 6.1 MG/KG*; PH RAISED THE DOSE TO 3.5 MG/KG*; WHILE NF RAISED THE DOSE TO 4.2 MG/KG*. COMBINING AP AND PH PRODUCED CPN AT 8.7 MG/KG* WHILE COMBINING AP AND NF PRODUCED CPN AT 9.4 MG/KG*. NEITHER V NOR ATROPINE METHYLBROMIDE, 1.0 MG/KG, PRODUCED SIGNIFICANT PROTECTION.

THESE STUDIES INDICATE THAT DFP'S TOXIC EFFECTS ON RESPIRATION ARE DUE TO EFFECTS PRIMARILY AT CENTRAL, RATHER THAN PERIPHERAL SITES. THE INCREASE IN PROTECTION OVER THE SCP PRODUCED BY PH AND NF IS PROBABLY DUE TO BOTH CENTRAL AND PERIPHERAL ACTIONS OF THESE DRUGS. THE LACK OF PROTECTION OF V AND ATROPINE METHYLBROMIDE IS PROBABLY DUE TO POOR ENTRY INTO THE CENTRAL NERVOUS SYSTEM. IT IS CONCLUDED THAT THE CALCIUM CHANNEL BLOCKING AGENTS MAY BE A VALUABLE ADJUNCT TO AP AND 2-PAM IN PREVENTING ORGANOPHOSPHATE TOXICITY.

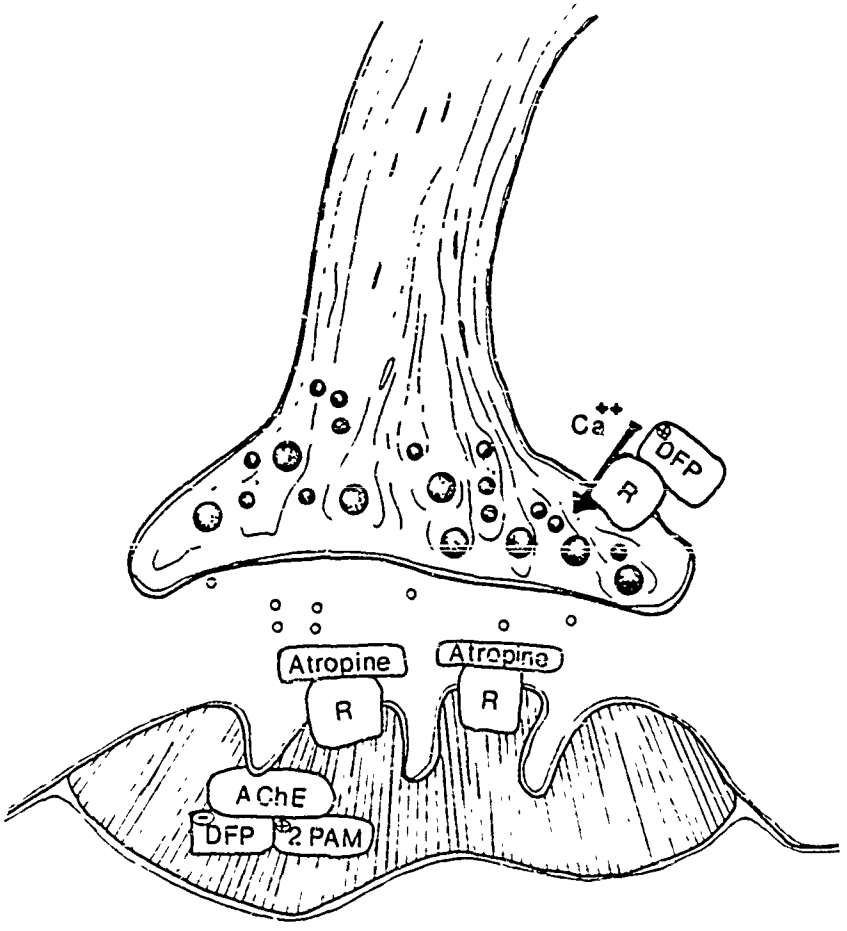
* DIFFERENT FROM CONTROLS ($P < 0.05$)

THIS WORK SUPPORTED IN PART BY NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND UNDER CONTRACT NO. N0014-83-K-0047.

INTRODUCTION

ORGANOPHOSPHATES, SUCH AS DIISOPROPYLFLUOROPHOSPHATE (DFP), ARE RESPONSIBLE FOR HUNDREDS OF INTOXICATIONS ANNUALLY IN THE UNITED STATES THROUGH THEIR WIDESPREAD USE AS INSECTICIDES. THE STOCKPILING OF THESE AGENTS FOR POTENTIAL USE IN WARFARE REPRESENTS AN EVEN GREATER THREAT TO MILITARY AND CIVILIAN PERSONNEL.

CURRENTLY, MAXIMUM PROTECTION AGAINST THE TOXIC EFFECTS OF THESE AGENTS IS PROVIDED BY THE COMBINED ACTIONS OF ATROPINE (AP) AND 2-PRALIDOXIME (2-PAM). IN ADDITION TO INHIBITING CHOLINESTERASE, ORGANOPHOSPHATES PROMOTE PRESYNAPTIC RELEASE OF ACETYLCHOLINE, PERHAPS BY INCREASING CALCIUM INFLUX AT CHOLINERGIC SYNAPSES. THE PURPOSE OF THESE STUDIES WAS TO EVALUATE THE DEGREE OF PROTECTION CALCIUM CHANNEL BLOCKING AGENTS AFFORDED IN THE PRESENCE OR ABSENCE OF THE STANDARD COMBINED PRETREATMENT (SCP) OF AP AND 2-PAM.



A-796

Protective Effects of Atropine and 2-PAM Against DFP Toxicity in Mice

Pretreatment	Dose mg/kg	DFP LD_{50} mg/kg
None	--	6.0
Atropine SO_4	1.0	7.3[a]
2-PAM	10.0	8.1[a]
Atropine plus 2-PAM	1.0; 10.0	8.7[a,b]

a = Significantly greater than LD_{50} DFP alone; $P < 0.05$

b = Significantly greater than LD_{50} DFP plus Atropine or 2-PAM; $P < 0.05$

Protective Effects of Calcium Antagonists Against DFP Toxicity in Mice

Pretreatment	LD_{50} mg/kg Alone	LD_{50} mg/kg with SCP
Verapamil 3.0 mg/kg	7.5[a]	--
Verapamil 2.5 mg/kg	--	9.3
Phenytoin 15.0 mg/kg	6.8[a]	--
Phenytoin 25.0 mg/kg	--	10.6[b]
Nifedipine 0.05 mg/kg	8.8[a]	--
Nifedipine 0.10 mg/kg	--	13.6[b]

SCP = Standard Combined Pretreatment (Atropine 1.0 mg/kg + 2-PAM 10.0 mg/kg i.p.)

a = Significantly greater than LD_{50} alone, $P < 0.05$

b = Significantly greater protection than SCP alone; $P < 0.05$

THE RESULTS OF THE LD_{50} STUDIES INDICATE THAT CALCIUM CHANNEL ANTAGONISTS, SUCH AS NIFEDIPINE AND PHENYTOIN, ENHANCE THE PROTECTIVE EFFECTS OF THE SCP. HOWEVER, THESE STUDIES FAIL TO LOCALIZE THE SITE AT WHICH THE CALCIUM CHANNEL BLOCKERS EXERT THEIR PROTECTION. IS IT CENTRAL, PERIPHERAL OR BOTH? IN ORDER TO DETERMINE THIS, WE EVALUATED THE EFFECTS OF DFP ON EEG AND NEUROMUSCULAR ACTIVITIES SIMULTANEOUSLY IN CATS.

METHODS

1) CATS WERE ANESTHETIZED WITH ALPHA-CHLORALOSE (80 MG/KG).

2) A FEMORAL VEIN WAS CANNULATED FOR I.V. ADMINISTRATION OF AGENTS.

3) NEUROMUSCULAR ACTIVITY WAS MEASURED VIA INDIRECT STIMULATION OF THE GASTROCNEMIUS MUSCLE.

4) GROSS EEG ACTIVITY WAS MONITORED USING SCREW ELECTRODES IMPLANTED BILATERALLY IN THE PARIETAL BONE.

5) CENTRALLY MEDIATED RESPIRATORY ACTIVITY WAS MEASURED AS FOLLOWS: PHRENIC NEURAL FIRING WAS MEASURED IN FREE BREATHING CATS. THE PHRENIC NERVE WAS ISOLATED, DESHEATHED, AND PLACED ON BIPOLAR, PLATINUM, RECORDING ELECTRODES. PHRENIC DISCHARGE WAS INTEGRATED.

6) CALCIUM ANTAGONISTS AND ATROPINE WERE INTRODUCED I.V. PRIOR TO DFP.

7) DFP WAS ADMINISTERED I.V. IN INCREMENTAL DOSES AND THE AMOUNT OF DFP NECESSARY FOR CESSATION OF EEG, PHRENIC, AND NEUROMUSCULAR ACTIVITY WAS DETERMINED.

CAT DFP RESPONSES

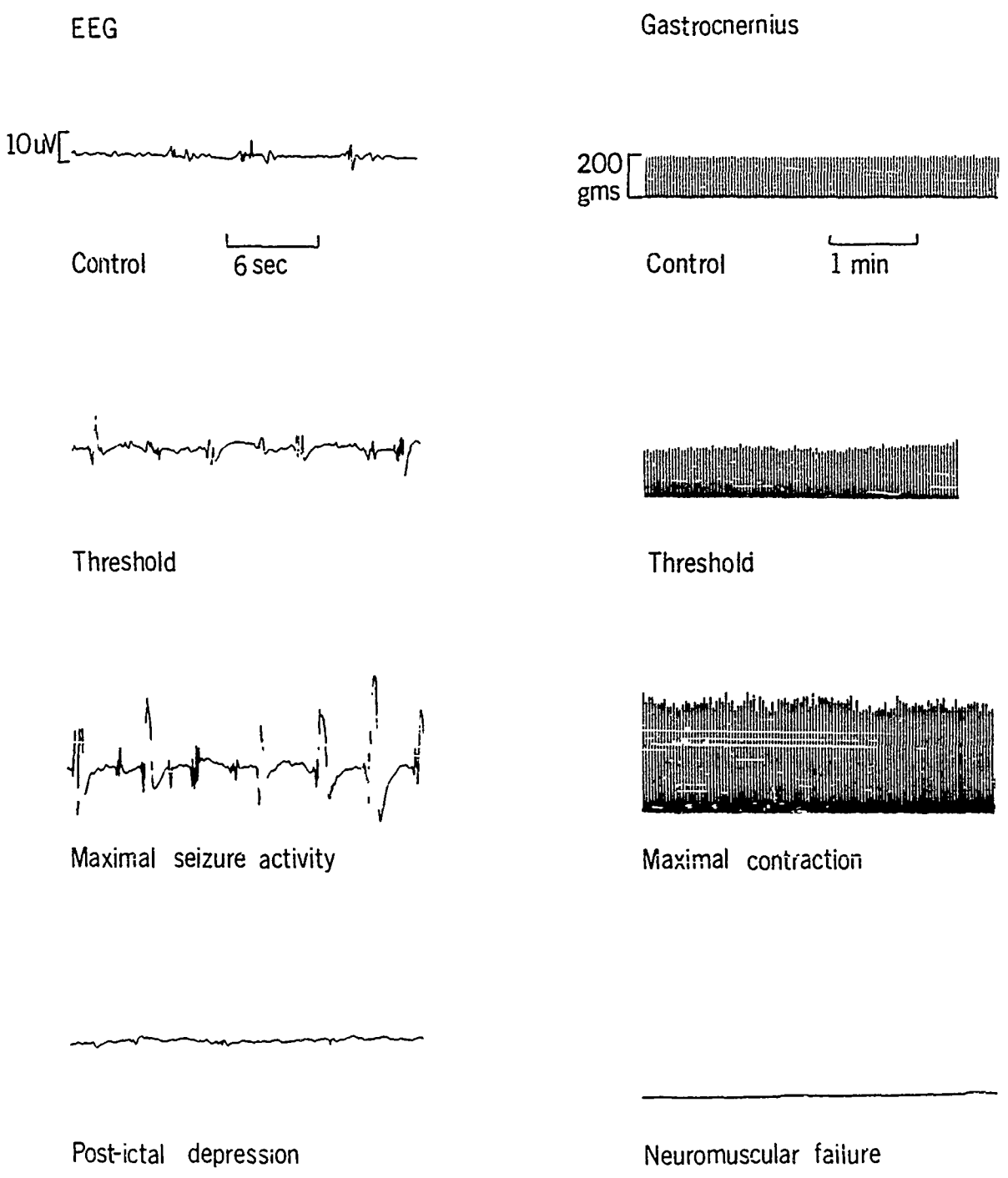


FIGURE 1: EFFECT OF DFP ON EEG AND NEUROMUSCULAR ACTIVITIES IN THE CAT. THE TRACES REPRESENT THE RESPONSES TO INCREMENTAL DOSES OF DFP ON BOTH PARAMETERS.

INTERACTION OF ATROPINE AND CALCIUM ANTAGONISTS ON DFP ACTIVITY IN THE CAT

Gastrocnemius Muscle Contractility

Treatment	Threshold	Neuromuscular Failure
DFP Alone	1.0 mg/kg iv*	4.2
DFP + Atropine (1mg/kg)	1.4	4.4
DFP + Phenytoin (25mg/kg)	1.2	8.4
DFP + Atropine + Phenytoin	1.4	9.0
DFP + Verapamil (1mg/kg)	1.3	5.2

EEG

Treatment	Threshold	Maximal Seizure Activity
DFP Alone	1.4	3.6
DFP + Atropine	4.2	10.0
DFP + Phenytoin	2.8	4.8
DFP + Atropine + Phenytoin	4.5	16.2
DFP + Verapamil	1.5	2.9

*Dose of DFP necessary to produce response, mean of 5 cats

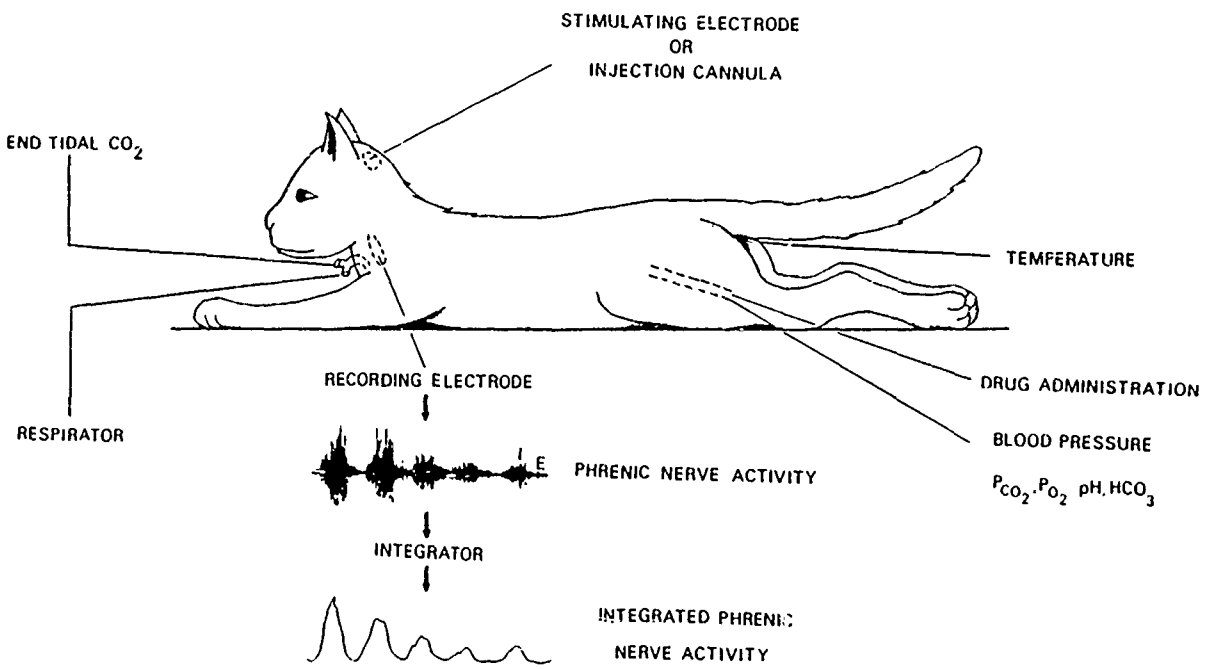


FIGURE 2: ILLUSTRATION OF THE TECHNIQUE USED TO RECORD PHRENIC NERVE BURSTING ACTIVITY IN THE CAT. THE NEURAL DISCHARGE WAS INTEGRATED IN ORDER TO QUANTIFY THE RESPONSE.

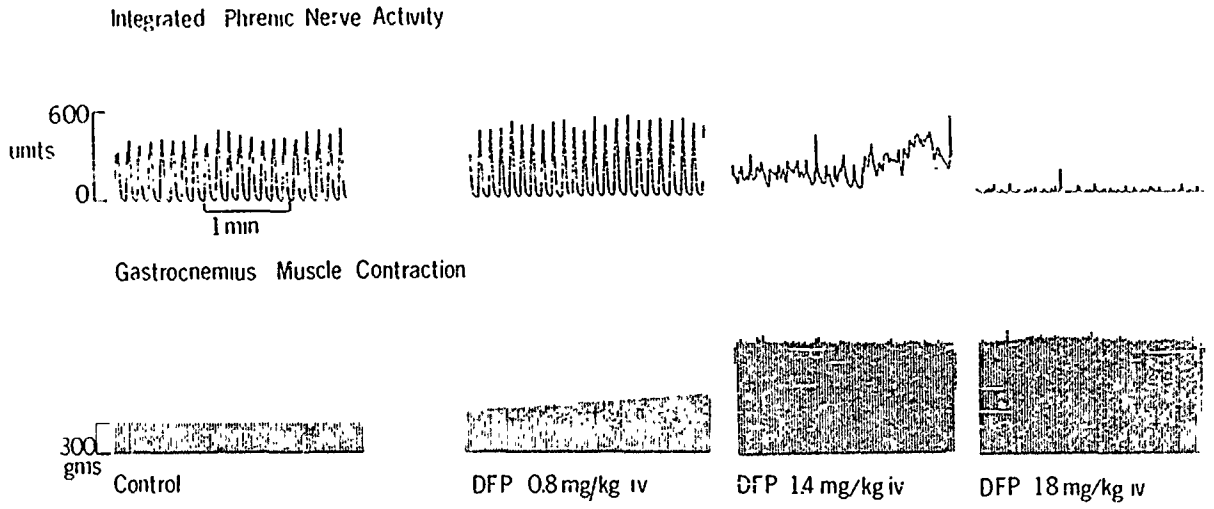


FIGURE 3: TYPICAL RECORD OF EFFECT OF DFP ON INTEGRATED NERVE ACTIVITY AND SKELETAL MUSCLE CONTRACTION IN THE CAT.

Doses of DFP needed to inhibit phrenic nerve bursting activity

TREATMENT	DOSE OF DFP (mg/Kg)
Control	2.3 ± .27 (S.E.)
Atropine (1 mg/Kg)	6.1 ± .26
Atropine methyl bromide	3.3 ± .36
Verapamil (1 mg/Kg)	2.8 ± .28
Phenytoin (25 mg/Kg)	3.5 ± .26
Nifedipine (0.05 mg/Kg)	4.2 ± .45
Phenytoin and Atropine	8.7 ± .44
Nifedipne and Atropine	9.4 ± 1.7

SUMMARY OF RESULTS

1) EEG VS. NEUROMUSCULAR ACTIVITY: MAXIMAL SEIZURE ACTIVITY WAS INDUCED WITH LOWER DOSES OF DFP THAN THOSE NEEDED TO BLOCK NEUROMUSCULAR TRANSMISSION. PRETREATMENT WITH ATROPINE AND PHENYTOIN PROVIDED PROTECTION AGAINST THE TOXIC EFFECTS OF DFP, WHILE VERAPAMIL WAS NOT EFFECTIVE.

2) INHIBITION OF PHRENIC NERVE BURSTING ACTIVITY:

A) DFP DEPRESSED PHRENIC BURSTING AT DOSES AT WHICH NEUROMUSCULAR TRANSMISSION WAS NOT REDUCED. PRETREATMENT WITH ATROPINE, PHENYTOIN AND NIFEDIPINE PROVIDED PROTECTION AGAINST THIS EFFECT.

B) VERAPAMIL DID NOT SIGNIFICANTLY PROLONG PHRENIC ACTIVITY.

C) ATROPINE METHYL BROMIDE SHOWED MINIMAL PROTECTIVE EFFECTS AGAINST THE DFP INHIBITION OF RESPIRATORY ACTIVITY.

Effect of Sensory Deafferentation on Dose of DFP
Needed to Inhibit Phrenic Nerve Bursting Activity

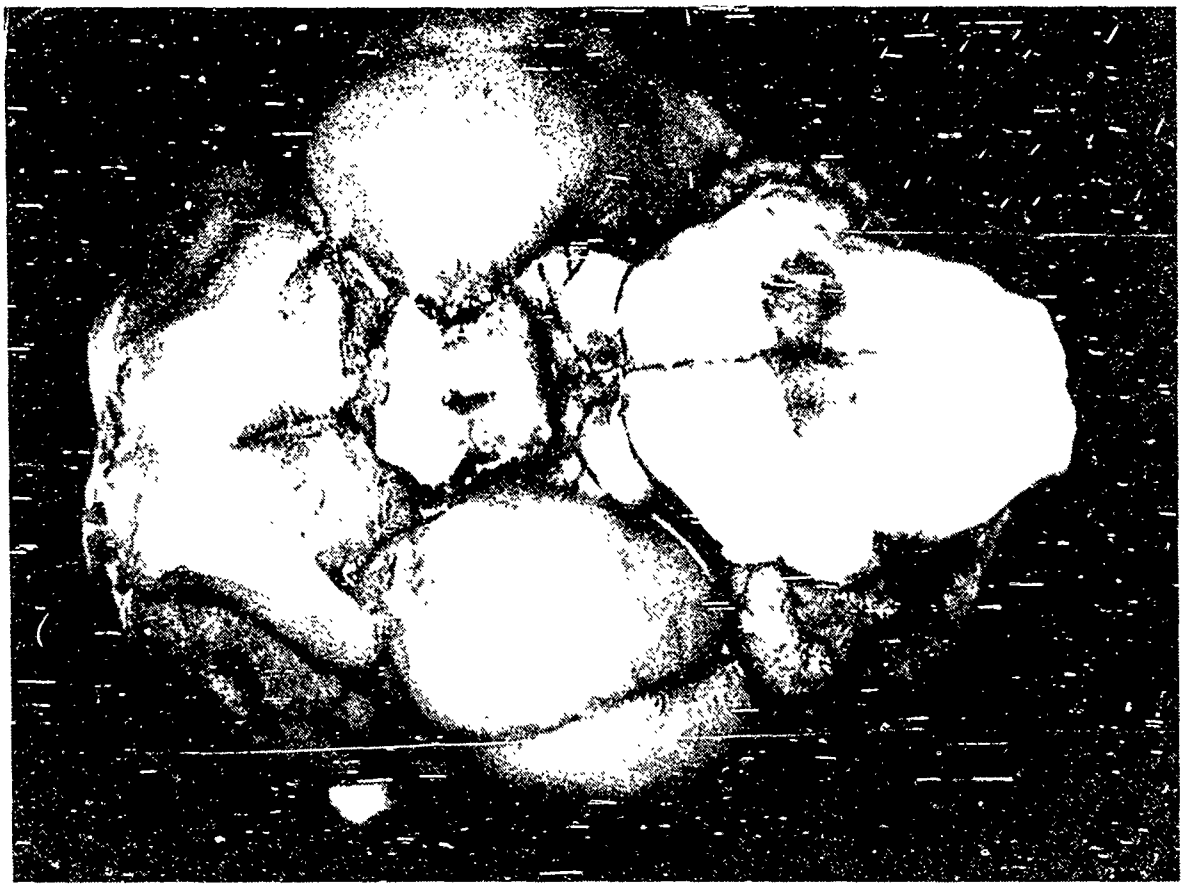
Treatment	Dose of DFP (mg/kg)
Transected Carotid Sinus Nerves	2.6 ± .233
Transected Vagus Nerves at Nodose Ganglia	2.6 ± .204
Thoracotomy with Phrenic Nerves Transected at Insertion into Diaphragm	3.9 ± .252
Thoracotomy with use of Pancuronium	3.6 ± .462

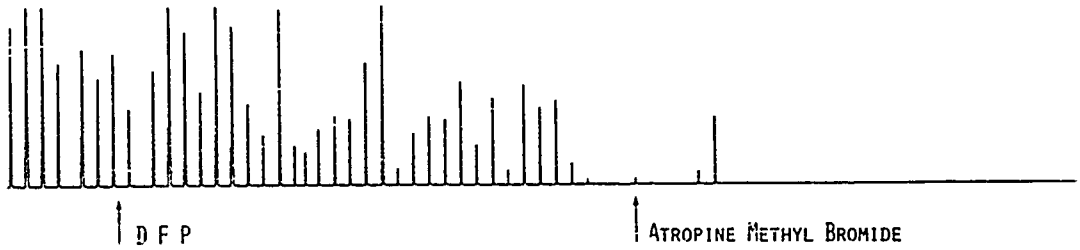
All values are Mean ± S.E.

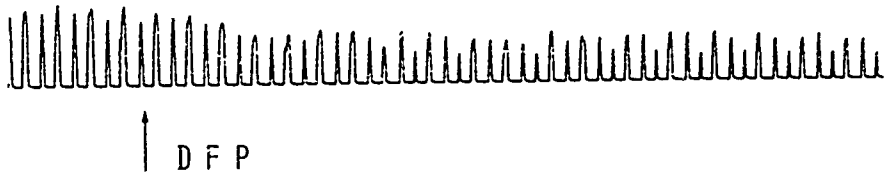
Effect of Various Treatment Parameters on the Dose of Neostigmine
Needed to Inhibit Phrenic Nerve Bursting Activity

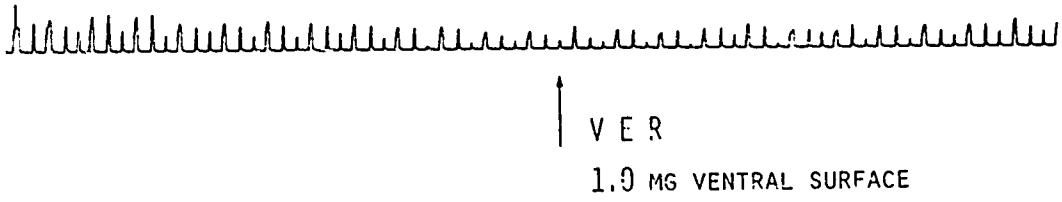
Treatment	Dose of Neostigmine (mg/kg)
Control	.17 ± .012
Atropine (1.0 mg/kg)	.14 ± .019
Transected Carotid Sinus Nerves	.20 ± .027
Transected Vagus Nerves at Nodose Ganglia	.40 ± .098
Thoracotomy with Phrenic Nerves Transected at Insertion into Diaphragm	1.3 ± .223
Thoracotomy with use of Pancuronium	.70 ± .250

All values are Mean ± S.E.




D F P
1.8 MG/KG I.V.
ATROPINE METHYL BROMIDE
1.9 MG/KG I.V.


D F P
1.8 MG/KG I.V.


V E R
1.9 MG VENTRAL SURFACE

CONCLUSIONS

1) THE TOXIC EFFECT OF DFP TO DEPRESS RESPIRATION IS DUE TO A CENTRAL, RATHER THAN A PERIPHERAL ACTION.

2) THE CALCIUM CHANNEL ANTAGONISTS AND OTHER PROTECTIVE AGENTS THAT PENETRATE THE BLOOD-BRAIN BARRIER AFFORD THE MOST PROTECTION AGAINST DFP TOXICITY.

3) THE PROTECTION PROVIDED BY THE CALCIUM CHANNEL BLOCKERS IS PROBABLY MEDIATED AT CENTRAL RESPIRATORY CENTERS.

CHOLINE TRANSPORT AND METABOLISM IN BRAIN EXPOSED TO AN ORGANOPHOSPHATE CHOLINESTERASE INHIBITOR

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Department of Biochemistry - School of Medicine
University of Minnesota, Duluth, Minnesota 55812

ABSTRACT

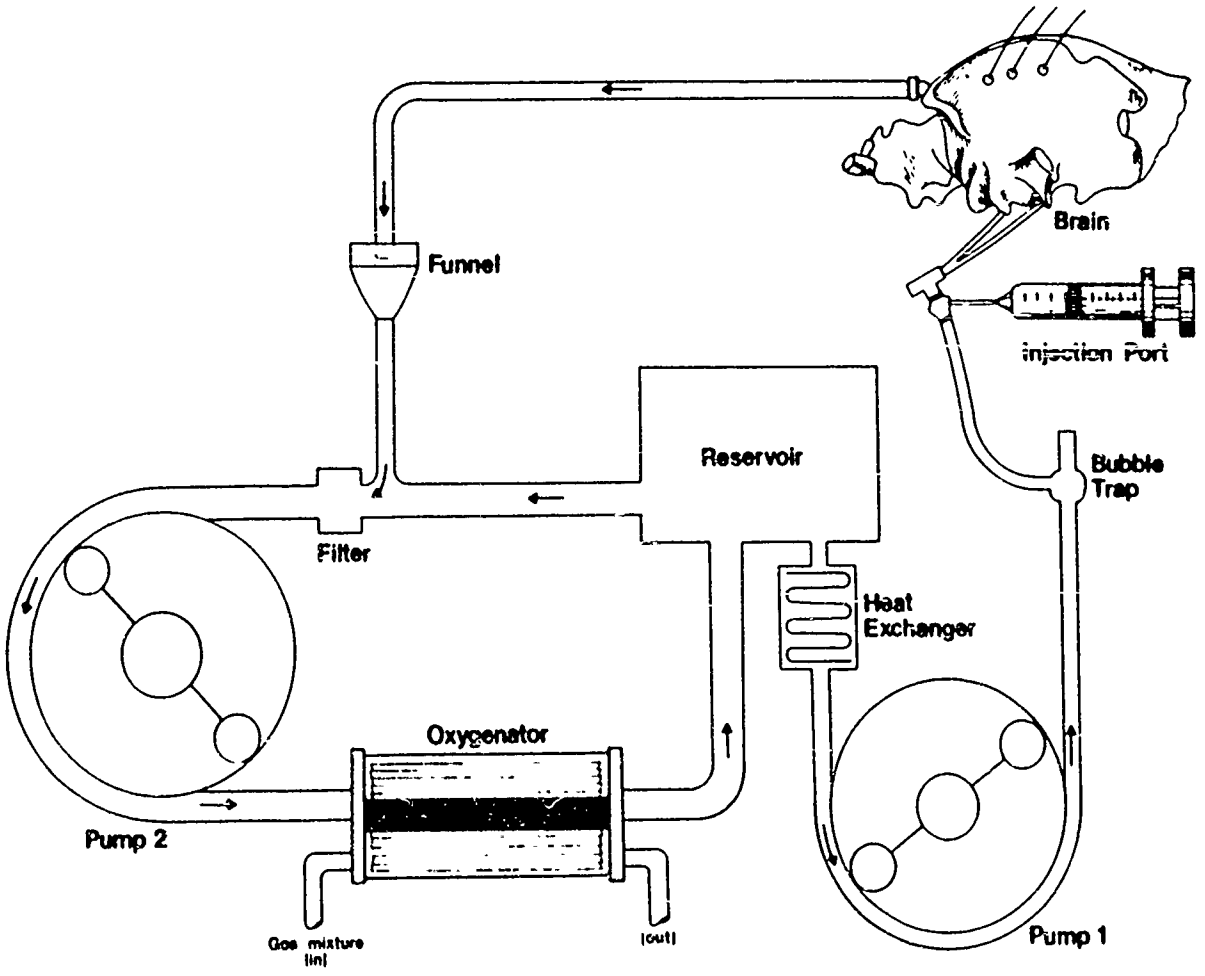
The effects of an organophosphate (OP) on brain acetylcholine (ACh) and choline (Ch) levels, net Ch transport, and unidirectional blood-brain Ch transport in the isolated, perfused canine brain were investigated. The brain was perfused with heparinized blood (hematocrit 33%) at constant flow (0.65 ml/g•min) for periods up to 90 minutes. Arterial and venous samples were collected at frequent intervals to measure pH and blood gases and for analysis of Ch, glucose, and lactate. After each experiment brain samples were rapidly collected from the cerebral cortex, cerebellum, hippocampus, and brainstem for analysis of ACh and Ch. After a control period of approximately 30 minutes, soman (100 µg) was administered by rapid injection into a carotid cannula. Within 5 minutes brain tissue concentrations of both Ch and ACh were increased by 100 to 500%. Regional differences in levels and responses were observed for ACh. At 60 minutes after soman exposure, the ACh values in brain cortex and brainstem were not significantly different from the 5-minute values, whereas in the hippocampus the amount of ACh was twice the 5-minute value. At 60 minutes after soman exposure, the Ch levels were slightly reduced below the 5-minute values. Net choline efflux of 1.51±0.20 nmol/g•min was observed during control perfusion periods. This efflux decreased significantly to 0.32±0.07 nmol/g•min following OP exposure. Unidirectional transport of Ch at the blood-brain barrier was unaltered by the cholinesterase inhibitor, thus indicating that the choline carrier of endothelial cells is unaffected by OP. Net choline efflux from brain suggests that choline is a net product of brain metabolism. The brain's high metabolic ability to form or accumulate choline is also indicated by the rapid rise in tissue concentrations of Ch and ACh after OP exposure. The source of this choline is unknown; however, blood-brain transport of choline and the choline precursors ethanolamine and serine is insufficient to account for the increased tissue choline. It is proposed that choline efflux normally occurs from the pool of extracellular choline. After OP exposure, extracellular choline decreases as a result of acetylcholinesterase inactivation and neuronal uptake of choline, thus causing a decline in choline efflux. It is further proposed that intracellular choline simultaneously is produced from endogenous sources such as phospholipids.

(This work was supported in part by the US Army Medical Research and Development Command under Contract DAMD-17-82-C-2136.)

OBJECTIVES

The objectives of this investigation were to study the effects of organophosphorus compounds, sarin and soman, on (1) acetylcholine and choline levels, (2) unidirectional choline transport and (3) net choline efflux in the isolated perfused canine brain.

METHODS



PERFUSION PROTOCOL

TIME: 0 - 120 minutes with continuous recording of EEG and perfusion pressure

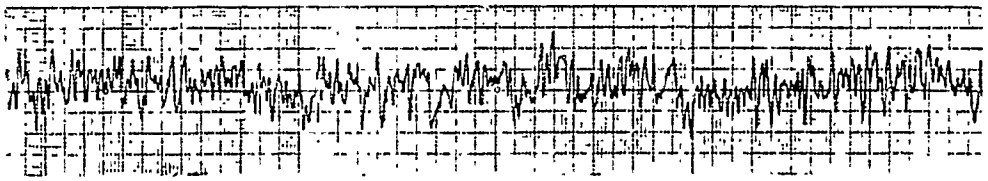
BLOOD SAMPLES: Collected every 10 minutes for:
(1) Glucose (arterial & venous)
(2) Choline (arterial & venous)
(3) Blood gases (arterial & venous)
(4) Plasma cholinesterase (venous)
(5) RBC cholinesterase (venous)

INDICATOR DILUTION: Immediately prior to OP exposure
Immediately after OP exposure
30 minutes after OP exposure

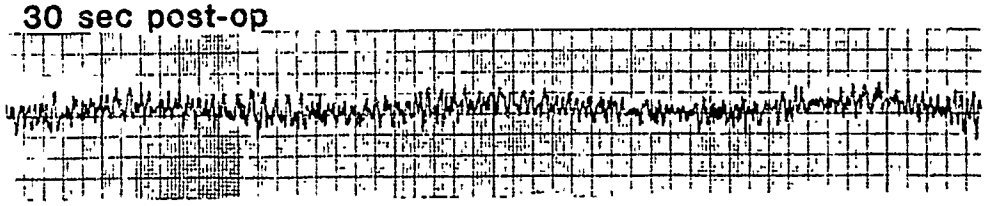
END OF PERFUSION: Brain removed and dissected into regions and assayed for:
(1) AChE
(2) Choline
(3) Acetylcholine

RESULTS

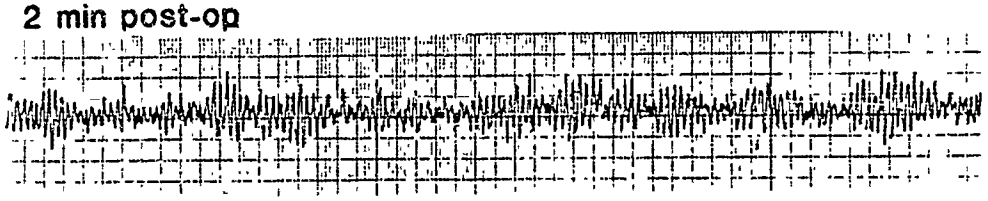
control



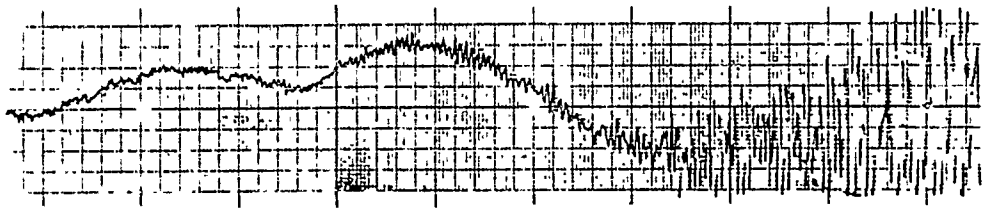
30 sec post-op



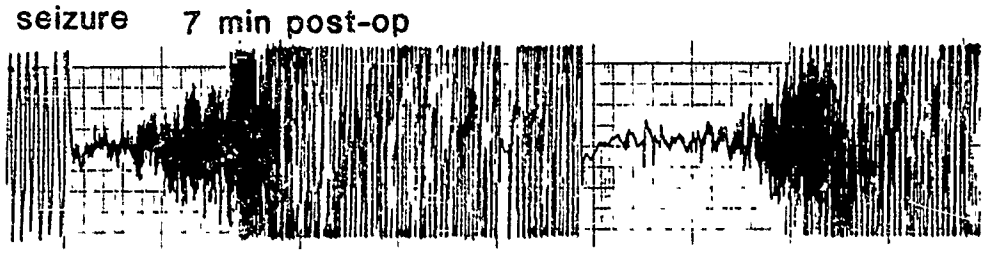
2 min post-op



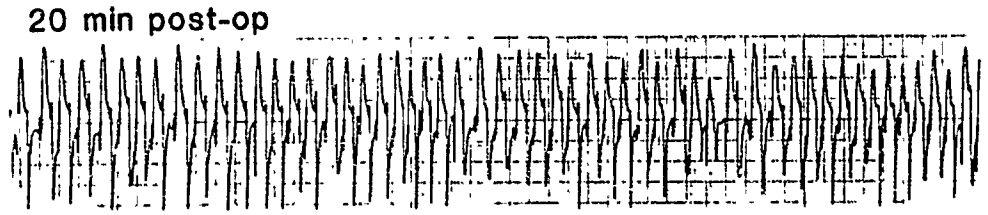
initial seizure 5 min post-op



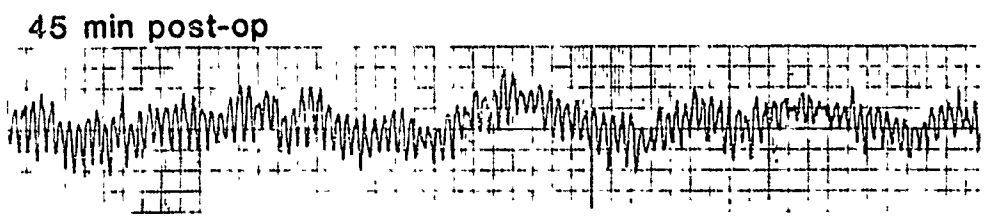
seizure 7 min post-op



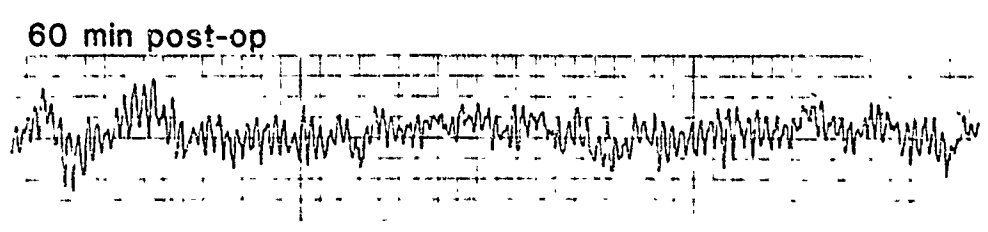
20 min post-op

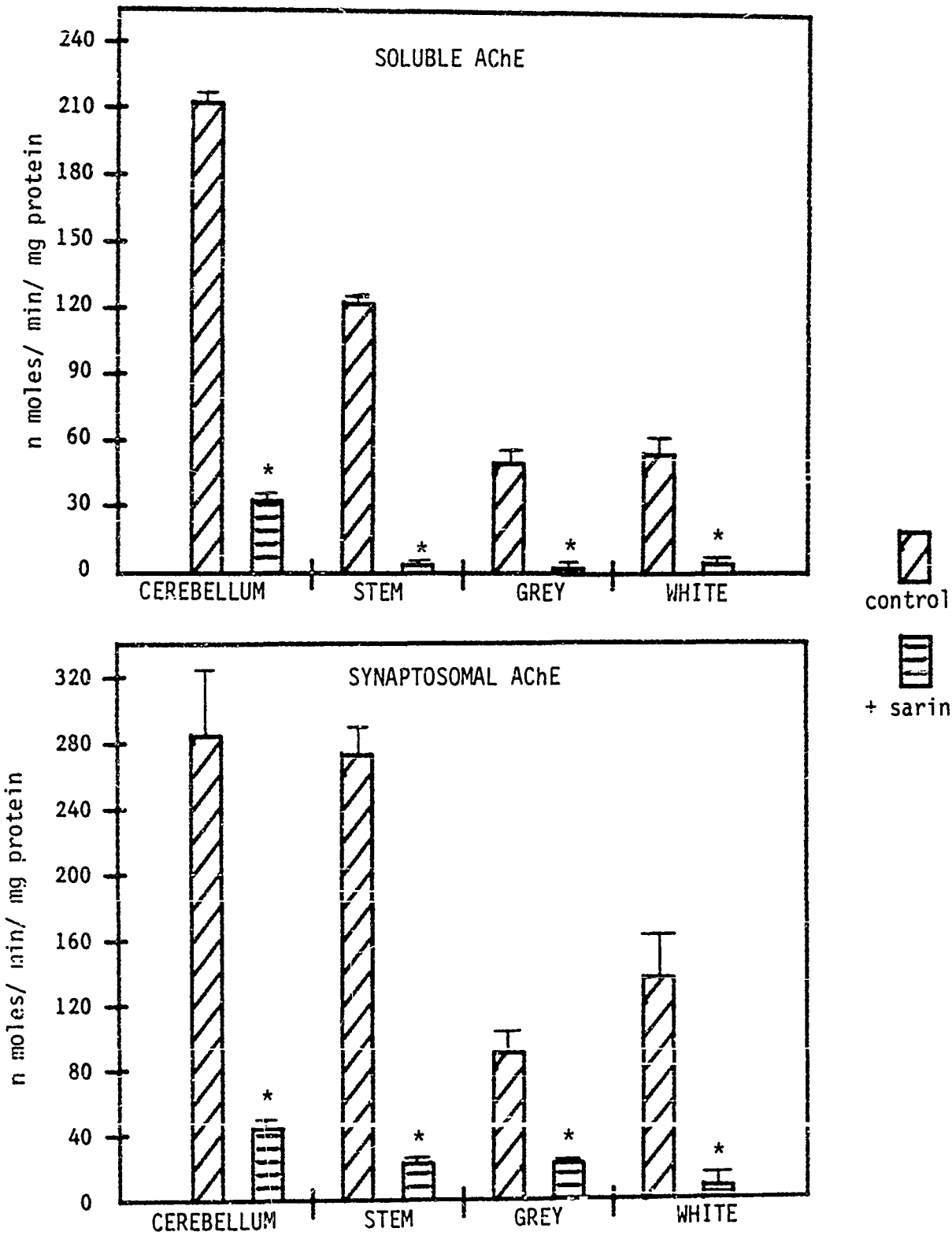


45 min post-op



60 min post-op




SOLUBLE AChE
n moles/ min/ mg protein
240
210
180
150
120
90
60
30
0
CEREBELLUM
STEM
GREY
WHITE
SYNAPTOSOMAL AChE
n moles/ min/ mg protein
320
280
240
200
160
120
80
40
0
CEREBELLUM
STEM
GREY
WHITE
control
+ sarin

* $p < 0.05$

ACh AND Ch CONCENTRATIONS IN NON-PERFUSED AND ISOLATED PERFUSED CANINE BRAIN.

Values are mean ±SD, n=3.

Brain region	ACh (nmol/g)		Ch (nmol/g)	
	Non-perfused brain	Isolated perfused brain	Non-perfused brain	Isolated perfused brain
Cerebral cortex	4.33±0.67	7.50±2.30	40.97±2.07	288.00±170.00
Cerebellum	0.55±0.17	0.50±0.10	60.90±7.24	273.00±53.00
Hippocampus	1.23±0.28	0.80±0.20	67.30±5.30	250.00±32.00
Brain stem	4.0±0.21	2.6 (n=1)	78.3±2.42	254.00 (n=1)

Effect of soman administration on acetylcholine levels in isolated, perfused canine brain.

Brain region	Acetylcholine (nmols/g)	
	Control	Soman
Cerebral cortex	7.5 ± 2.3	66.9 ± 12.4*
Cerebellum	0.5 ± 0.1	1.6 ± 1.3
Hippocampus	0.8 ± 0.2	40.6 ± 8.8*
Brain stem	2.6 (n = 1)	53.9 ± 1.1*

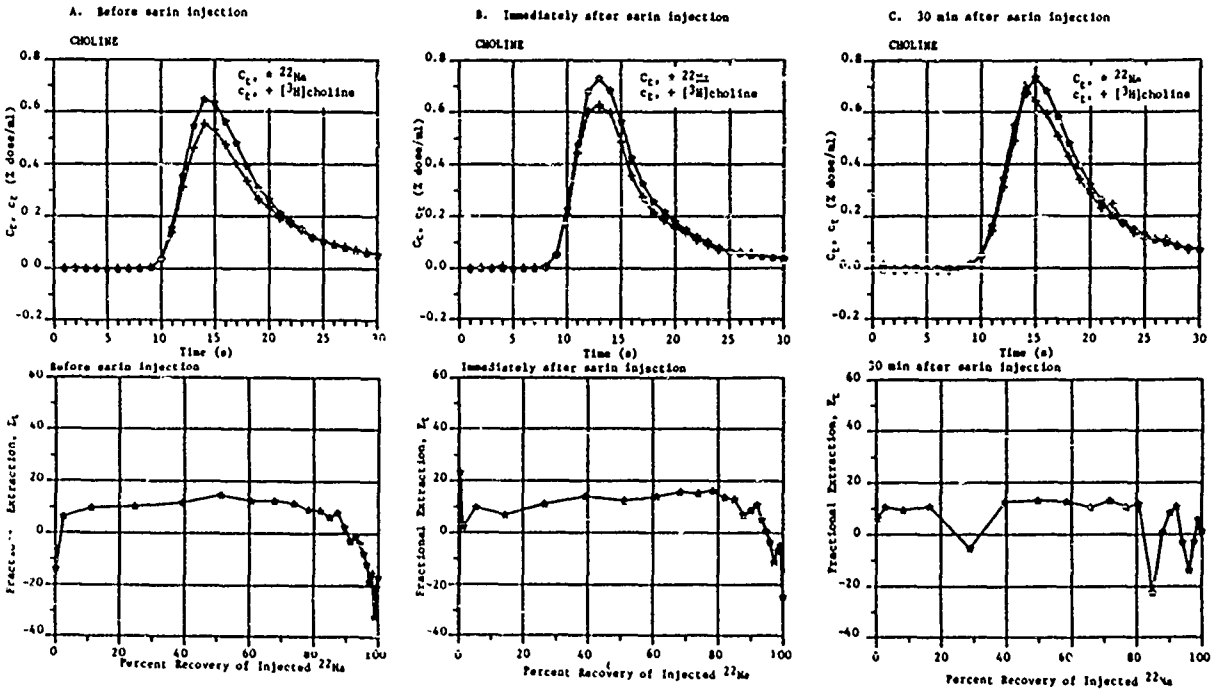
*p <0.05 when compared with control values, n = 3

Effect of soman administration on choline levels in isolated, perfused canine brain.

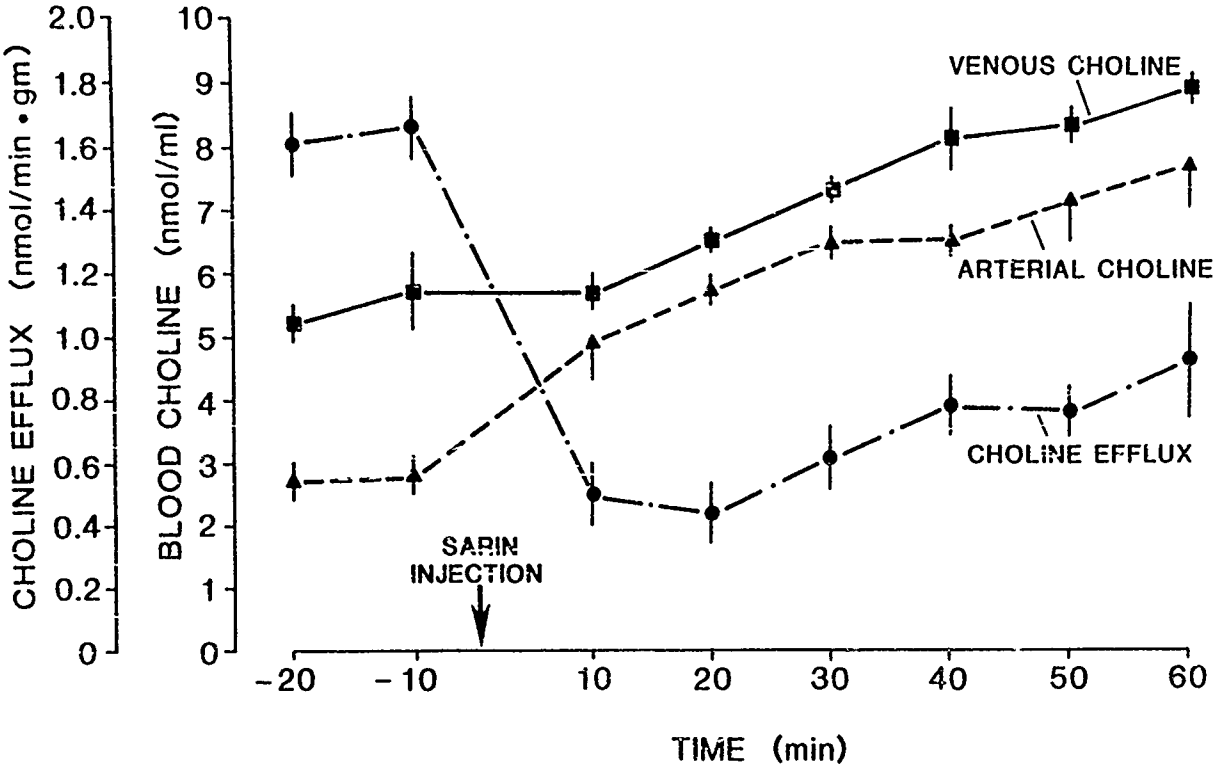
Brain region	Choline (nmols/g) Control	Soman
Cerebral cortex	288 ± 70	499 ± 18*
Cerebellum	273 ± 53	713 ± 43*
Hippocampus	250 ± 32	579 ± 26*
Brain stem	254 (n = 1)	839 ± 380*

*p <0.05 when compared to control. n = 3

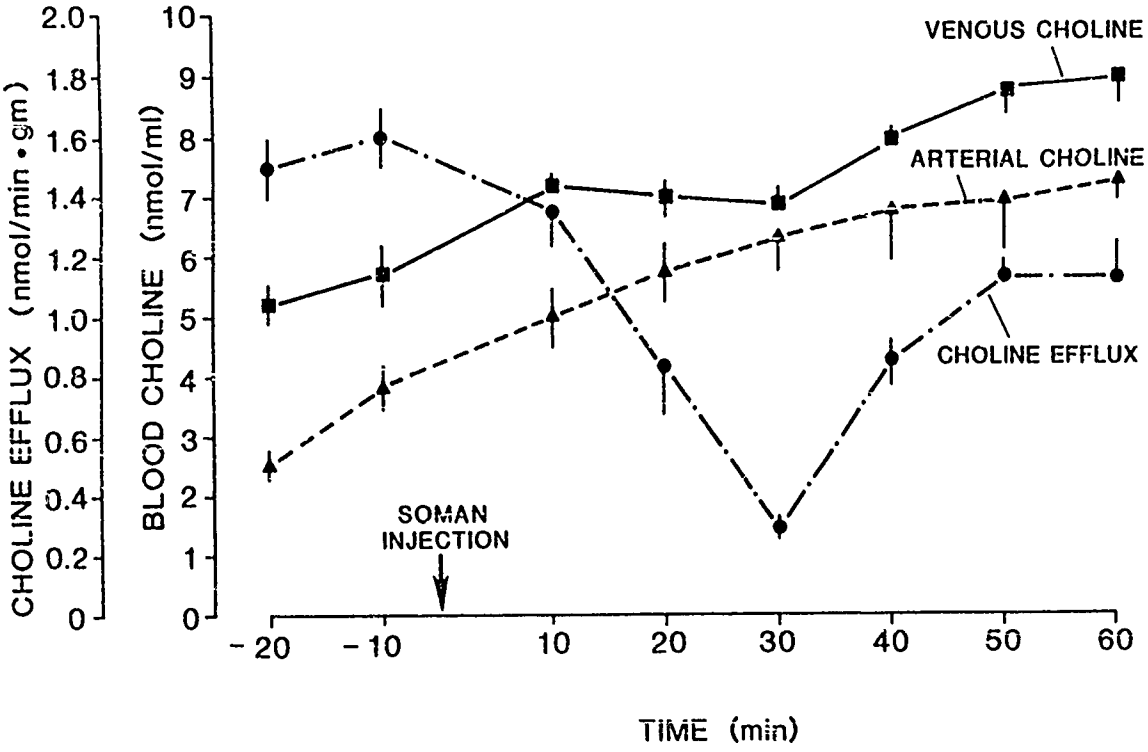
Unidirectional uptake of choline by the brain.



Effect of sarin administration on arterial and venous choline levels and net choline efflux in isolated, perfused canine brain.



Effect of soman administration on arterial and venous choline levels and net choline efflux in isolated, perfused canine brain.



CONCLUSIONS

1. Brain choline and acetylcholine level were increased by 100–500 per cent within 5 minutes after exposure to sarin or soman. Regional differences in levels and responses were observed for ACh.

2. The unidirectional transport of choline to the brain was unaltered by these compounds.

3. We observed net efflux of choline (1.51 ± 0.2 nmol/g min) during control perfusion. This efflux decreased 30 minutes after OP exposure to 0.32 ± 0.07 nmol/g min.

4. These results indicate that blood choline is not the source of elevated brain levels of choline after OP exposure. It is proposed that choline is derived from endogenous sources by a mechanism involving a receptor–mediated process.

INTERACTION OF REVERSIBLE AND IRREVERSIBLE CHOLINESTERASE (ChE) INHIBITORS WITH THE NICOTINIC AND GLUTAMATERGIC SYNAPSES

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ABSTRACT

REVERSIBLE CHOLINESTERASE (ChE) INHIBITORS SUCH AS PYRIDOSTIGMINE (PASCUZZO et al., MOL. PHARMACOL. 25, 92, 1984) AND PHYSOSTIGMINE (SHAW et al., NEUROSCI. ABS. 10,562, 1984) INTERACT WITH THE ACh RECEPTOR-ION CHANNEL COMPLEX (AChR) TO REDUCE CHANNEL CONDUCTANCE AND ENHANCE DESENSITIZATION OF THE VERTEBRATE SKELETAL MUSCLE ENDPLATE. IN ADDITION, THESE DRUGS ACT AS WEAK AGONISTS, AND PHYSOSTIGMINE SHORTENS MEAN CHANNEL OPEN TIME. THE PRESENT REPORT DESCRIBES THE INTERACTION OF THE IRREVERSIBLE ChE INHIBITOR VX AT THE NEUROMUSCULAR JUNCTIONS OF FROG AND LOCUST. ENDPLATE CURRENTS (EPCs), MINIATURE ENDPLATE CURRENTS (MEPCs), AND EPC FLUCTUATIONS (NOISE) WERE RECORDED FROM THE ENDPLATE OF THE SARTORIUS MUSCLES OF FROG AND EXTENSOR OR FLEXOR TIBIALIS MUSCLES OF LOCUSTS. SINGLE CHANNEL CURRENTS WERE RECORDED USING PATCH CLAMP TECHNIQUE FROM THE PERIJUNCTIONAL REGION OF THE FROG INTEROSSEAL MUSCLES. VX (0.1 μM) INCREASED THE PEAK AMPLITUDE 27% AND THE DECAY TIME CONSTANT (τ_{EPC}) 129% AT -80 mV. VX CONCENTRATION > 1 μM CAUSED A VOLTAGE-DEPENDENT DECREASE IN THE PEAK AMPLITUDE AND SHORTENED τ_{EPC}. THIS FINDING SUGGESTS THAT VX INTERACTS DIRECTLY WITH THE AChR. THE CHANNEL LIFETIME WAS SIGNIFICANTLY DECREASED, SUGGESTING THAT VX BLOCKS CHANNELS IN OPEN CONFORMATION. WHEN LOCUST NEUROMUSCULAR JUNCTION WAS EXPOSED TO VX, TABUN AND DFP, THE INCREASED SPONTANEOUS NEUROTRANSMITTER RELEASE WAS LARGE ENOUGH TO TRIGGER ACTION POTENTIALS (APs) AND ENDPLATE POTENTIALS (EPPs) IN A CYCLIC PATTERN OF BURSTS AND SILENCE. REDUCTION OF THE EXTERNAL Ca^{++} CONCENTRATION ($[Ca^{++}]_o$) TO 0.8 mM ABOLISHED APs BUT NOT EPPs. A FURTHER REDUCTION OF $[Ca^{2+}]_o$ TO 0.3 mM BLOCKED BOTH APs AND EPPs, SUGGESTING A PRESYNAPTIC SITE OF ACTION OF OP COMPOUNDS. THE POSSIBILITY THAT THESE OP AGENTS INDUCED A TRANSIENT PRESYNAPTIC DEPOLARIZATION COULD EXPLAIN THE REPETITIVE EPPs AND THE INCREASED SPONTANEOUS TRANSMITTER RELEASE. IF THE APPEARANCE OF SPONTANEOUS EPPs INVOLVED A PARTIAL INCREASE IN SODIUM CONDUCTANCE, AGENTS SUCH AS TETRODOTOXIN SHOULD BLOCK THIS EFFECT OF THE OP COMPOUNDS. IN THE PRESENCE OF OP AGENTS, TETRODOTOXIN (0.3 μM) COMPLETELY BLOCKED THE SPONTANEOUS ACTIVITY, WHICH RETURNED WHEN THE PREPARATION WAS WASHED. SIMILAR TO THE FROG NEUROMUSCULAR JUNCTION, EXPOSURE OF THE LOCUST MUSCLES TO VX RESULTED IN SHORTENING OF τ_{EPC}, AND A DECREASED PEAK AMPLITUDE WITH A MARKED NONLINEARITY IN THE CURRENT VOLTAGE RELATIONSHIP. NOISE ANALYSIS REVEALED A SIGNIFICANT SHORTENING OF CHANNEL LIFETIME IN PRESENCE OF VX (10 μM). IN CONCLUSION, AT THE NICOTINIC SYNAPSE OF FROG MUSCLE, VX CAUSES AChE INHIBITION AND A SHORTENING OF CHANNEL LIFETIME. THE ACTION OF VX ON THE GLUTAMATERGIC SYNAPSE OF LOCUST MUSCLES DEMONSTRATES PRESYNAPTIC AND POSTSYNAPTIC ACTIONS OF VX ON A NONCHOLINERGIC SYSTEM. THESE OBSERVATIONS COULD EXPLAIN SOME OF THE CONVULSIONS AND SEIZURES FREQUENTLY SEEN IN EXPERIMENTAL ANIMALS EXPOSED TO NERVE AGENTS. (SUPPORTED BY U.S. ARMY MED. RES. AND DEVELOP. COMMAND CONTRACT DAMD-17-81-C-1279).

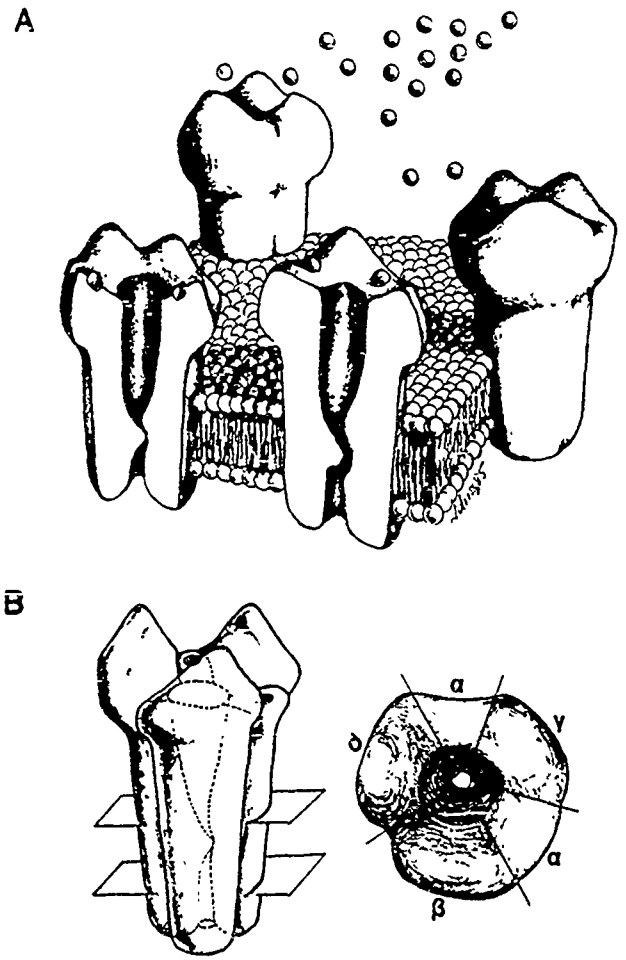
INTRODUCTION

THE ACTIONS OF REVERSIBLE AND IRREVERSIBLE ChE INHIBITORS AT THE NICOTINIC ACETYLCHOLINE RECEPTOR-IONIC CHANNEL (AChR) ARE FAR MORE COMPLEX THAN IT HAS BEEN THOUGHT. RECENT STUDIES (1,2, AND 3) IN THIS LABORATORY HAVE SHOWN THAT ASIDE FROM INHIBITING ChE, SOME OF THESE AGENTS SUCH AS PYRIDOSTIGMINE (PYR), PHYSOSTIGMINE (PHY) AND NEOSTIGMINE (NEO) ACT AS AGONISTS AND INCREASE THE AFFINITY OF ACETYLCHOLINE (ACh) TO ITS BINDING SITE, ENHANCING ACTIVATION AND DESENSITIZATION OF THE NICOTINIC AChR. PHY, IN ADDITION, ACTS AS A NONCOMPETITIVE BLOCKER THROUGH INTERACTION WITH SITES AT THE IONIC CHANNEL IN ITS OPEN CONFORMATION. THE NOTION THAT ChE IS NOT THE ONLY TARGET FOR THESE AGENTS IS FURTHER STRENGTHENED BY RECENT OBSERVATIONS (4) THAT BOTH CARBAMATE AND ORGANOPHOSPHORUS (OP) ChE INHIBITORS ALSO AFFECT PRE- AND POST-SYNAPTIC REGIONS OF THE LOCUST NEUROMUSCULAR JUNCTION (A GLUTAMATERGIC SYNAPSE).

STUDIES IN RATS (5) INDICATED THAT PHY WAS AN EFFECTIVE DRUG IN PROTECTING AGAINST LETHAL EFFECT OF SARIN. THE PROTECTION OFFERED BY PHY WAS FURTHER ENHANCED BY CO-ADMINISTRATATION OF A MUSCARINIC ANTAGONIST ATROPINE (ATR) AND A GANGLIONIC BLOCKING DRUG, EITHER MECAMYLAMINE (MEC) OR CHLORISONDAMINE (CHL).

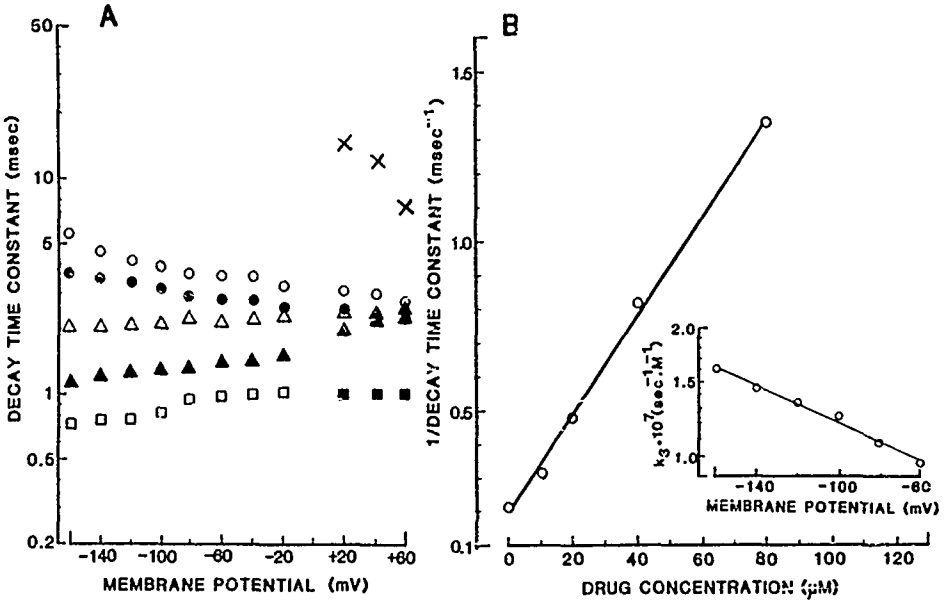
THE PURPOSE OF THE PRESENT INVESTIGATION WAS:

1. TO ANALYZE THE EFFECTS OF THE ANTI-ChE AGENTS WITH THE NICOTINIC AChR OF THE FROG MUSCLE AND ON THE GLUTAMATERGIC NEUROMUSCULAR SYNAPSE OF THE LOCUST MUSCLE.
2. TO PROVIDE A MOLECULAR BASIS FOR THE DEVELOPMENT OF PROPHYLACTIC DRUGS AGAINST LETHALITY OF OP AGENTS.



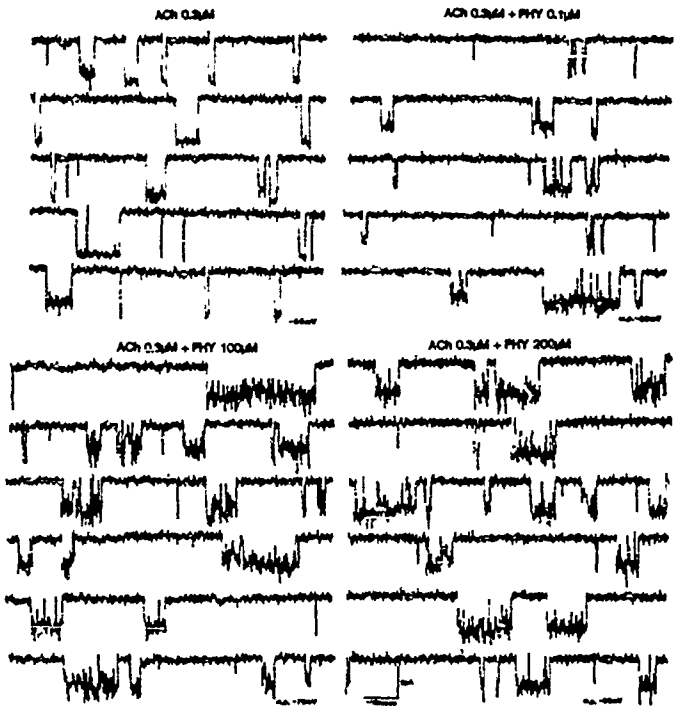
THREE-DIMENSIONAL VIEW OF THE NICOTINIC ACETYLCHOLINE RECEPTOR-ION CHANNEL COMPLEX.

A: NICOTINIC AChR MACROMOLECULES EMBEDDED IN THE POSTSYNAPTIC MEMBRANE IN CLOSED (LEFT FRONT) AND OPEN (RIGHT FRONT) CHANNEL CONFORMATIONS. SPHERES ATTACHED TO AND FLOATING OVER THE RECEPTOR PROTEIN ARE TO REPRESENT AGONIST MOLECULES. B: DIAGRAMMATIC REPRESENTATIONS OF THE PROFILE (LEFT) AND TOP-VIEW (RIGHT) OF THE AChR. THE TOPOGRAPHIC ARRANGEMENT OF THE RECEPTOR SUBUNITS SUGGESTED BY KARLIN et al. (6) IS ALSO SHOWN.



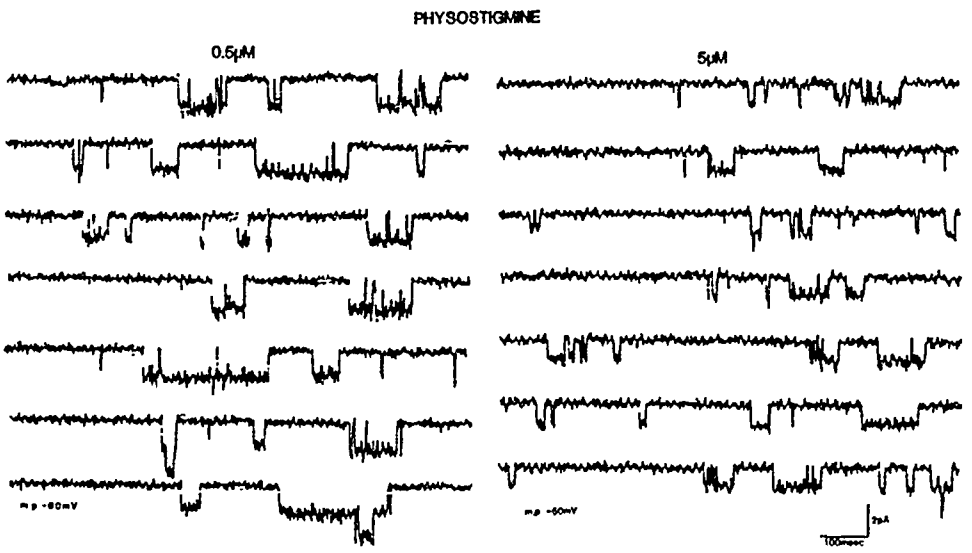
EFFECT OF PHYSOSTIGMINE ON THE DECAY TIME CONSTANT OF ENDPLATE CURRENTS RECORDED FROM FROG SARTORIUS MUSCLE PRETREATED WITH DFP.

A: VOLTAGE DEPENDENCE OF τ_{EPC} BEFORE (○) AND AFTER EXPOSURE OF THE MUSCLES TO 10 (●), 20 (△), 40 (▲) AND 80 (□) μM PHYSOSTIGMINE. (■) AND (×) REPRESENT, RESPECTIVELY, τ OF THE FAST AND SLOW COMPONENTS OF THE DOUBLE EXPONENTIAL DECAYS OBSERVED IN THE PRESENCE OF 80 μM PHY AT POSITIVE POTENTIALS. B: RECIPROCAL OF τ_{EPC} PLOTTED AGAINST CONCENTRATION OF PHY. INSET: EXPONENTIAL VOLTAGE DEPENDENCE OF THE FORWARD RATE CONSTANT OF THE BLOCKING REACTION (k_3). EACH POINT IS THE MEAN ± SEM OF 8 TO 24 SURFACE FIBERS FROM 2-6 MUSCLES. NOTE THE CONCENTRATION-DEPENDENT SHORTENING OF THE τ_{EPC}.



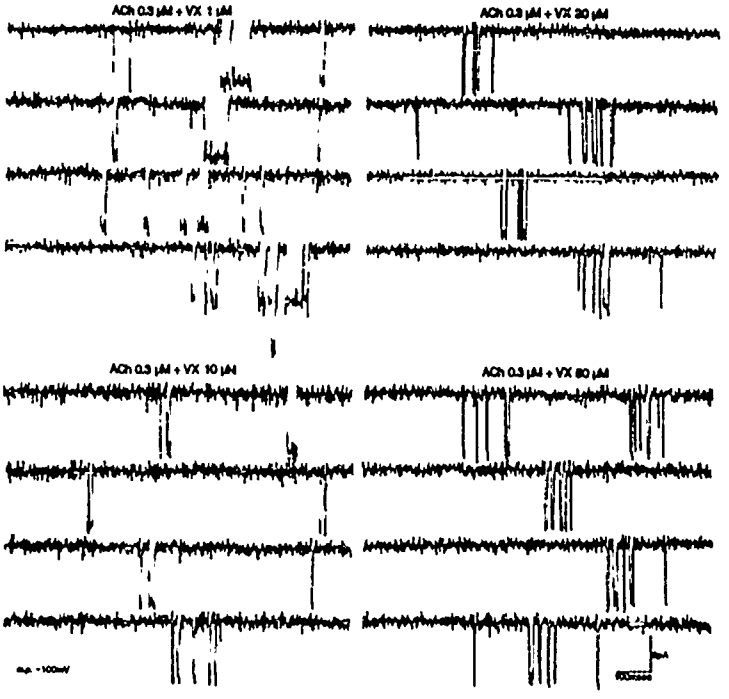
SAMPLES OF ACh-ACTIVATED SINGLE CHANNEL CURRENTS IN THE PRESENCE OF PHYSOSTIGMINE.

SINGLE CHANNEL CURRENTS WERE RECORDED FROM ISOLATED FIBERS OF THE FROG INTEROSSEAL MUSCLE. HIGH-RESISTANCE (GIGAOHM) SEALS WERE ACCOMPLISHED WITH A PIPETTE CONTAINING EITHER ACh (0.3 μM) ALONE OR IN COMBINATION WITH DIFFERENT CONCENTRATIONS (0.1 TO 200 μM) OF PHY. IN THE PRESENCE OF PHY, THE CHANNEL OPENINGS NO LONGER SHOWED A SQUARE SHAPE TYPICAL OF ACh-ACTIVATED CHANNEL CURRENTS.



SINGLE CHANNEL CURRENTS ACTIVATED BY PHYSOSTIGMINE.

PATCH ELECTRODES CONTAINED PHY (0.5 OR 5 μM) ALONE, AND THE CELL-ATTACHED RECORDS WERE OBTAINED FROM A SINGLE MUSCLE FIBER AT HOLDING POTENTIALS OF -50 AND -60 mV. NOTE THAT THE PHY ACTIVATED CHANNEL CURRENTS WERE SIMILAR TO THOSE GENERATED BY ACh TOGETHER WITH THIS AGENT.



SAMPLES OF ACh-ACTIVATED CHANNEL CURRENTS IN THE PRESENCE OF VX.

CHANNEL CURRENTS WERE RECORDED FROM SINGLE INTEROSSEAL MUSCLE FIBERS. THE PIPETTE WAS FILLED WITH ACh (0.3 μM) COMBINED WITH EITHER 1, 10, 20 OR 50 μM VX. THE ANALYSIS OF THE CLOSED INTERVALS WITHIN A BURST SHOWED AN ADDITIONAL SLOWER COMPONENT (15-20 msec) IN THE PRESENCE OF VX.

EFFECT OF VX ON MINIATURE ENDPLATE CURRENTS (MEPC) RECORDED FROM FROG SARTORIUS MUSCLES AFTER IRREVERSIBLE INHIBITION OF ChE BY DFP.

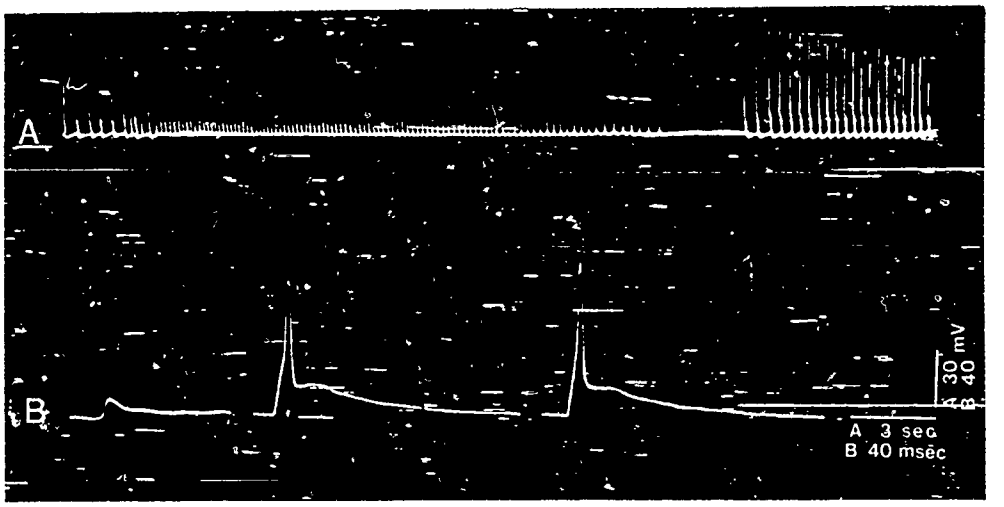
CONDITION	PEAK AMPLITUDE (nA)	DECAY TIME CONSTANT (τ_{MEPC}, msec)	N
CONTROL	9.8 ± 0.9	3.3 ± 0.4	118
10 μM VX	9.4 ± 0.9	3.1 ± 0.3	160
50 μM VX	8.6 ± 1.6	1.9 ± 0.2*	146
100 μM VX	6.8 ± 1.9	1.7 ± 0.2*	75
WASH (1 Hr)	7.6 ± 0.9	2.7 ± 0.2	81

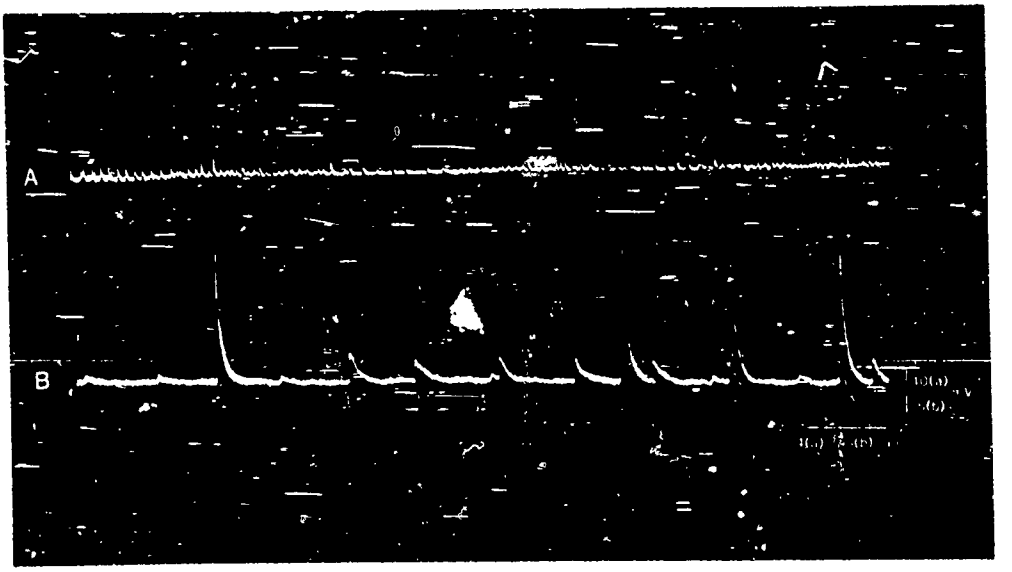
The values are mean ± SEM of MEPCs (N) recorded from at least 4 fibers in 4 muscles at -80mV holding potential.

ChE was inhibited by exposing muscles to DFP (1 mM) for 30 min and subsequently washing for 1hr.

*$P < 0.025$ as compared to control.

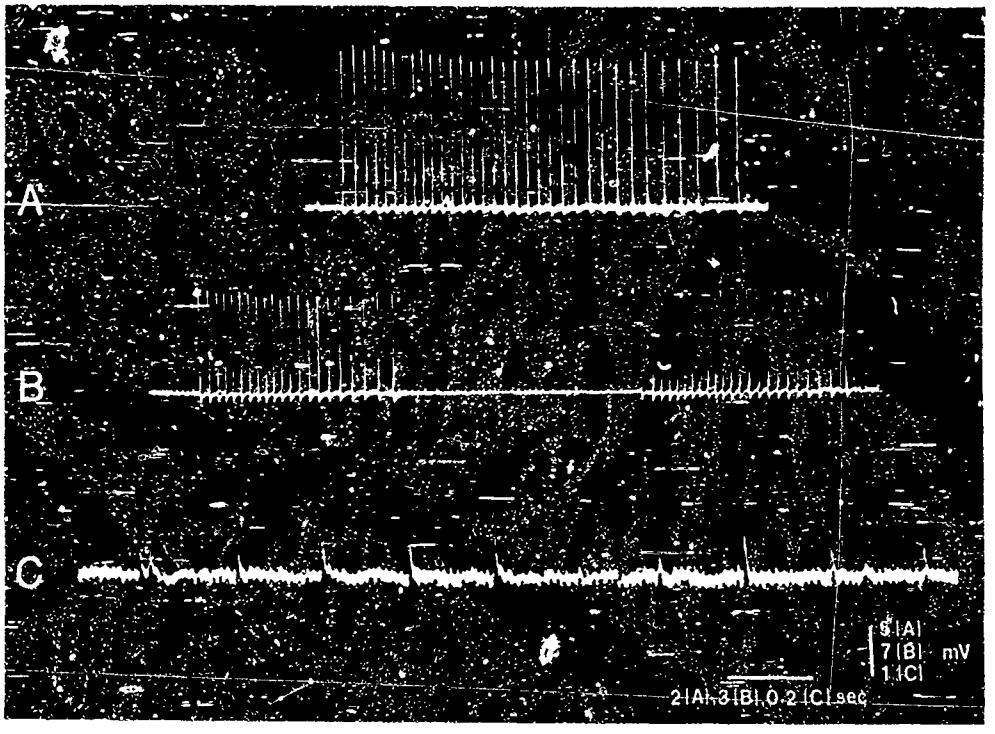
NOTE THE DOSE-DEPENDENT SHORTENING OF τ_{MEPC}.





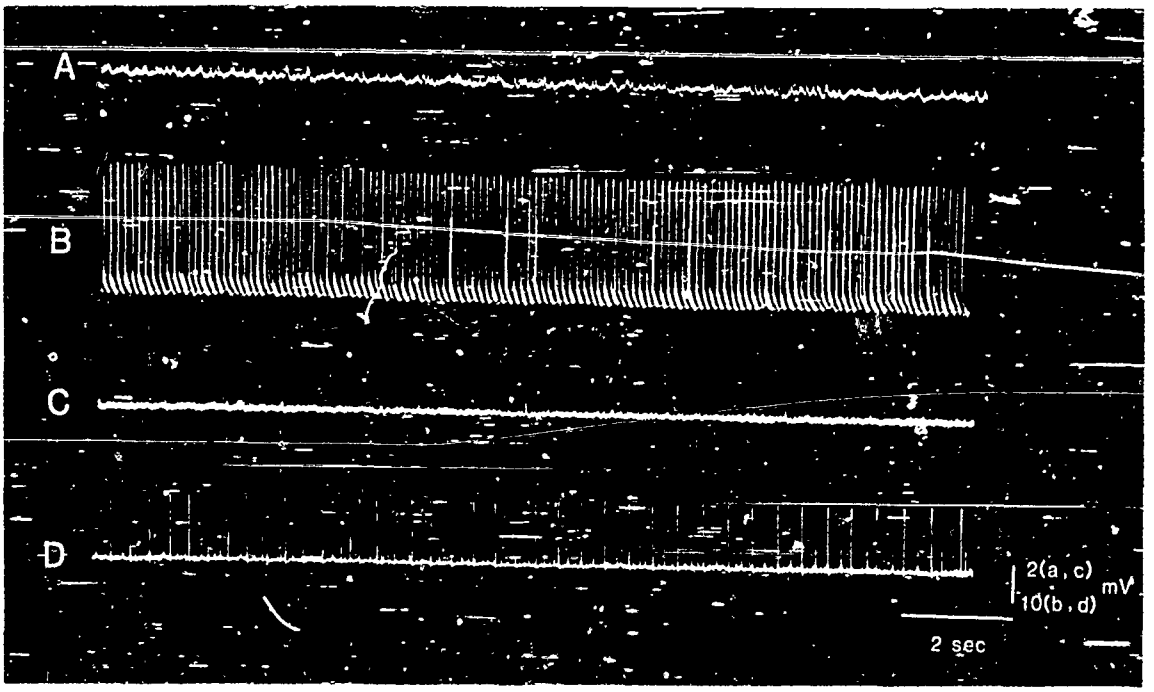
SPONTANEOUS APs AND EPPs RECORDED FROM LOCUST FLEXOR TIBIALIS MUSCLE IN THE PRESENCE OF DFP (0.5mM): EFFECT OF EXTERNAL [Ca^{2+}].

UPPER PANEL: SPONTANEOUS FIRING OF APs, EPPs AND SILENT PERIODS RECORDED IN THE PRESENCE OF DFP AND NORMAL EXTERNAL [Ca^{2+}] (2 mM). LOWER PANEL: SPONTANEOUS ACTIVITY IN THE PRESENCE OF REDUCED [Ca^{2+}] (0.8mM). NOTE THE ABSENCE OF THE APs. A FURTHER DECREASE IN [Ca^{2+}] TO 0.2mM BLOCKED THE EPPs. THESE DATA SUGGESTED AN INVOLVEMENT OF A Ca^{2+}-DEPENDENT PROCESS WHICH ULTIMATELY RESULTED IN INCREASED TRANSMITTER RELEASE. THIS HYPOTHESIS WAS STRENGTHENED BY FURTHER INVESTIGATION SHOWING A COMPLETE BLOCKADE OF THIS PROCESS BY TETRODOTOXIN.



SPONTANEOUS EPPs RECORDED FROM THE FLEXOR METATHORACIC TIBIALIS MUSCLE (FTiM) OF LOCUSTA MIGRATORIA EXPOSED TO 10 μM VX.

A: TYPICAL BURST OF EPPs AFTER 20-MIN EXPOSURE TO VX. NOTE THAT THE EPP FIRING FREQUENCY DECREASED TOWARDS THE END OF THE BURST. B: PATTERN OF BURSTS ALTERNATING WITH SILENT PERIODS. C: SPONTANEOUS MEPPs RECORDED DURING A SILENT PERIOD. Ca^{2+} CONCENTRATION IN THE PHYSIOLOGICAL SOLUTION BATHING THE MUSCLE WAS REDUCED FROM 2.0 mM TO 0.8 mM TO BLOCK SPONTANEOUS ACTION POTENTIALS. BOTH REVERSIBLE AND IRREVERSIBLE ANTI-ChE AGENTS GENERATED SIMILAR EFFECTS.



SPONTANEOUS ACTIVITY OF LOCUST FTIM IN THE PRESENCE OF TABUN.

A: CONTROL RECORDS SHOWING ONLY MEPPs; B: APs RECORDED AFTER 20-MIN EXPOSURE TO 20 μM TABUN; C: RECORDS AFTER MUSCLE EXPOSURE TO TABUN (20 μM) AND TTX (0.3 μM); D: RECORDS AFTER 60-MIN WASH WITH PHYSIOLOGICAL SOLUTION CONTAINING TABUN ALONE. THE REVERSIBLE BLOCKADE OF THIS ACTIVITY BY TTX SUGGESTED A PRESYNAPTIC SITE OF ACTION.

SUMMARY OF THE EFFECTS OF THE ANTI-ChE AGENTS ON THE ENDPLATE CURRENTS RECORDED FROM LOCUST GLUTAMATERGIC SYNAPSES.

AGENT	CONCENTRATION (μM)	DEPRESSION OF PEAK AMPLITUDE	SHORTENING OF τ_{EPC}
TABUN	1-100	-	-
PHYSOSTIGMINE	250-1000	+	-
VX	10-50	+	+
DFP	250-1000	+	+

+ denotes depression of the peak amplitude or shortening of the τ_{EPC}.

- denotes lack of effect.

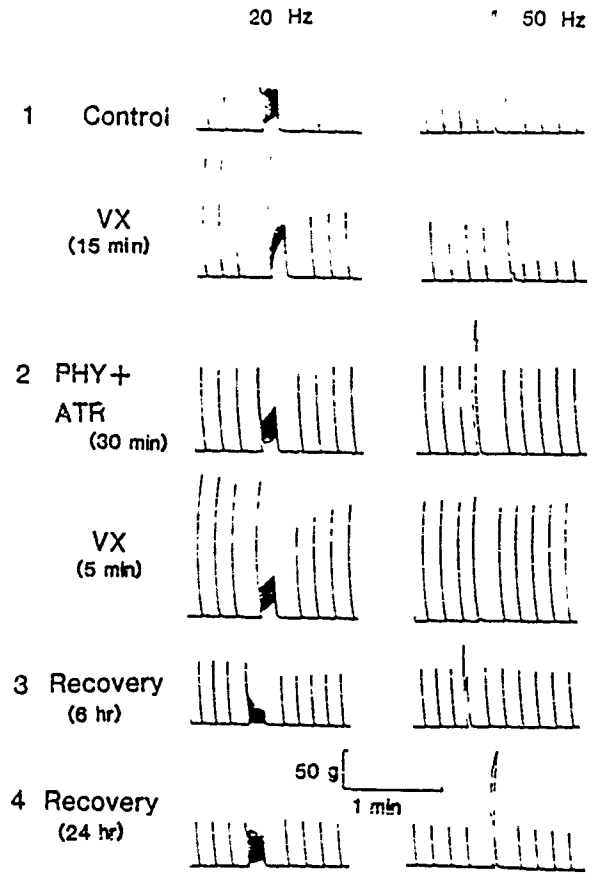
EFFECTIVENESS OF PRETREATMENT OF RATS WITH ATROPINE, MECAMYLAMINE, CHLORISONDAMINE AND PHYSCSTIGMINE IN PROTECTING AGAINST LETHALITY OF SUBCUTANEOUS INJECTIONS OF VX[A].

PRETREATMENT[b]	DOSE (mg/kg)	% LETHALITY[c]
NONE	-	100
ATROPINE AND MECAMYLAMINE	0.5 4.0	100
ATROPINE AND MECAMYLAMINE	0.5 8.0	100
ATROPINE AND CHLORISONDAMINE	0.5 0.5	100
ATROPINE AND PHYSOSTIGMINE	0.5 0.1	50
ATROPINE, MECAMYLAMINE AND PHYSOSTIGMINE	0.5 4.0 0.1	0
ATROPINE, CHLORISONDAMINE AND PHYSOSTIGMINE	0.5 2.0 0.1	0

[a] The minimal LD_{100} dose of VX was 0.015 mg/kg. The dose used in these experiments was 0.05 mg/kg, representing approximately 3.5 x LD_{100} Dose.

[b] All the drugs used in the pretreatment regimen were dissolved in 0.9% sodium chloride. The total intramuscular injection volume was 0.1 ml per 100 g body wt.

[c] The lethality is based on 24-hr observation in 6 rats per group.



IN VIVO RECORDS OF MUSCLE TWITCH AND TETANIC TENSIONS FROM RATS INJECTED WITH VX:

EFFECT OF PRETREATMENT WITH PHYSOSTIGMINE AND ATROPINE.

TWITCH AND TETANIC TENSIONS OF THE EXTENSOR DIGITORUM LONGUS MUSCLE WERE RECORDED FROM ANIMALS ANESTHETIZED WITH CHLORAL HYDRATE (400 mg/kg, IP). THE NERVE WAS CONTINUOUSLY STIMULATED AT 0.1 Hz, EXCEPT DURING 20 Hz (10 SEC) AND 50 Hz (2 SEC) REPETITIVE SUPRAMAXIMAL STIMULATION.

#1 - CONTROL: NOTE THE ABILITY OF THE MUSCLE TO MAINTAIN TENSION DURING 20 Hz AND 50 Hz STIMULATION. 15 MIN AFTER A SUBCUTANEOUS INJECTION OF VX (0.05 mg/kg): ABOUT A 3-FOLD POTENTIATION OF SINGLE TWITCH WAS OBSERVED, AND THE MUSCLE WAS UNABLE TO SUSTAIN A TETANUS AT 50 Hz. THIS ANIMAL DIED 17 MIN AFTER VX ADMINISTRATION.

#2 - 30 MIN AFTER A INTRAMUSCULAR INJECTION OF PHY (0.1 mg/kg) PLUS ATR (0.5 mg/kg): THIS TREATMENT INDUCED POTENTIATION OF THE SINGLE TWITCHES, AND TETANIC RESPONSE WAS SLIGHTLY DEPRESSED. 5 MIN AFTER VX (0.05 mg/kg): NOTE FURTHER POTENTIATION OF THE SINGLE TWITCHES ALONG WITH [illegible]BILITY OF THE MUSCLE TO SUSTAIN A TETANUS. NOTICE A POST TETANIC DEPRESSION OF THE TWITCH RESPONSE.

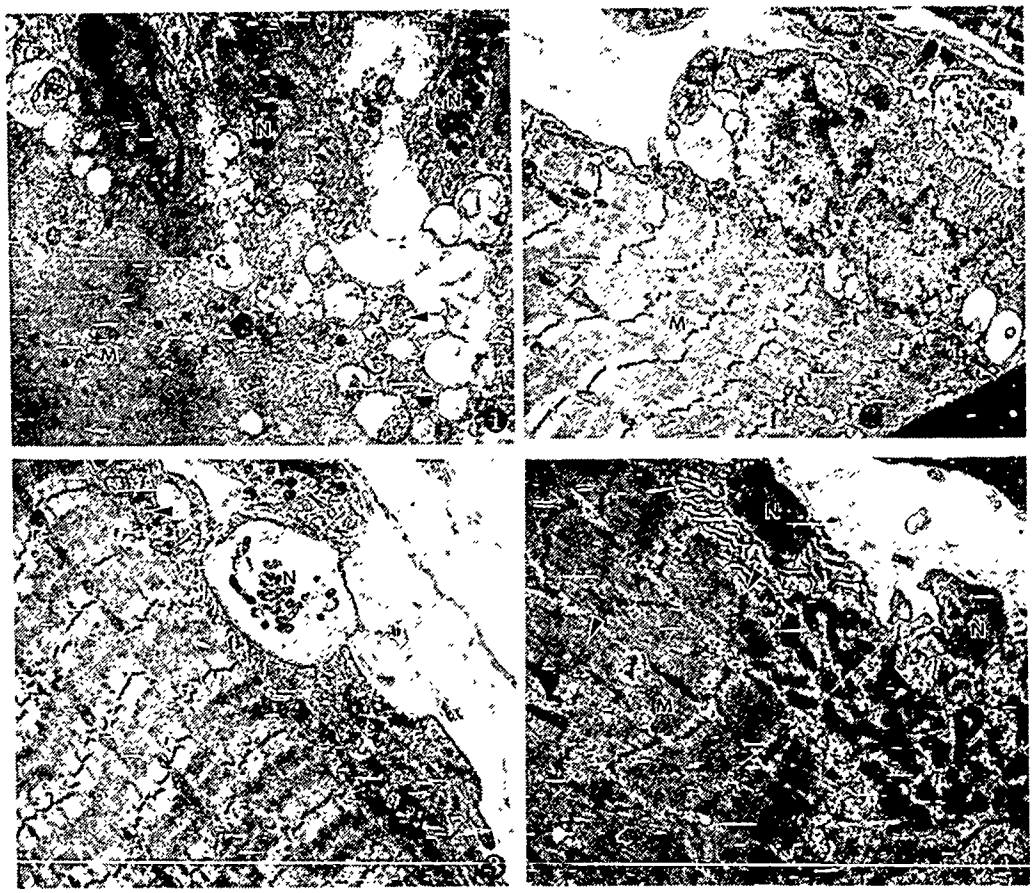
#3 - RECOVERY OF THE RESPONSES IN A RAT RECEIVING VX (0.05mg/kg) 30 MIN AFTER PREATREATMENT WITH PHY (0.1 mg/kg) + ATR (0.5mg/kg). THE CONTRACTIONS WERE RECORDED 6 Hr AFTER VX INJECTION.

#4 - RECOVERY AFTER 24 Hr. NOTE THAT THE MUSCLE RESPONSES RECOVERED NEARLY TO CONTROL CONDITIONS.

EFFECT OF PHYSOSTIGMINE AND ATROPINE PRETREATMENT AND SUBSEQUENT INJECTION OF SARIN ON BLOOD CHE AND SOLEUS MUSCLE AND BRAIN ACHE.

PRETREATMENT (IM, MG/KG)	SARIN (SUBCUTANEOUS, MG/KG)	PERCENT INHIBITION			PERCENT LETHALITY
		BLOOD CHE	MUSCLE ACHE	BRAIN ACHE	
NONE	0.13	87[A]	71	97	100
NONE	0.65	88	82	98	100
PHYSOSTIGMINE (0.1) + ATROPINE (0.5)	0.13	71	32	56	0
PHYSOSTIGMINE (0.1) + ATROPINE (0.5)	0.65	50	42	62	100

A PERCENT INHIBITION IS WITH RESPECT TO CONTROL VALUE IN SALINE INJECTED RATS BLOOD WAS COLLECTED FROM TAIL VEIN OF RATS LIGHTLY ANESTHETIZED WITH ETHER. SOLEUS MUSCLES AND BRAIN TISSUE (CEREBRAL HEMISPHERES) ACHE WAS ANALYSED USING MODIFIED ELLMAN ET AL. (8) PROCEDURE AND THE PROTEIN WAS DETERMINED BY THE METHOD OF LOWRY ET AL (9). NOTE LACK OF PROTECTION BY PHY + ATR IN RATS INJECTED WITH 5 X LETHAL DOSE OF SARIN. THESE RATS DIED INSPITE OF SIGNIFICANT PROTECTION OF CHE IN BLOOD AND ACHE IN THE MUSCLE AND BRAIN TISSUE BY PHY FROM SUBSEQUENT PHOSPHORYLATION BY SARIN.



ENDPLATE REGION OF SOLEUS MUSCLE FROM RATS ADMINISTERED SARIN WITH AND WITHOUT PHYSOSTIGMINE PRETREATMENT.

1. A RAT WAS INJECTED SUBCUTANEOUSLY WITH A SUBLETHAL DOSE OF SARIN (0.08 mg/kg). THE MUSCLE WAS REMOVED 1 Hr AFTER INJECTION. LONGITUDINALLY CUT EM SECTION SHOWS INTACT MOTOR NERVE TERMINALS (N). THE SOLEPLATE SARCOPLASM (S) IS DISTENDED AND IS FILLED WITH NUMEROUS LARGE VACUOLES OF MITOCHONDRIAL ORIGIN (ARROW). THE SUBJUNCTIONAL MYOFIBRILS (M) ARE COMPLETELY DISORGANIZED, LOSING THEIR ORIGINAL BANDING PATTERN.

2 & 3. ENDPLATE OF SOLEUS MUSCLE FROM A RAT PRETREATED WITH PHY (0.1 mg/kg) 30 MIN PRIOR TO A LETHAL INJECTION OF SARIN (0.13 mg/kg). THE MUSCLE WAS REMOVED 1 Hr AFTER SARIN INJECTION. NOTE A MARKED REDUCTION IN THE DEGREE OF MYOPATHIC LESIONS. THE BANDING PATTERN OF SUBJUNCTIONAL MYOFIBRILS (M) IS RELATIVELY WELL-PRESERVED (PANELS 2 & 3). IN SOME ENDPLATES LARGE VACUOLES OF MITOCHONDRIAL ORIGIN (ARROW IN 3) ARE SEEN IN THE AREA CONFINED TO SOLEPLATE SARCOPLASM (PANNEL 3). STATISTICAL ANALYSIS OF THE LIGHT MICROSCOPIC SECTIONS REVEALED THAT PHY PRETREATMENT CAUSED 27% REDUCTION IN THE AVERAGE LENGTH OF LESIONS AND 53% REDUCTION IN THE AVERAGE WIDTH OF LESIONS AFTER SARIN ADMINISTRATION.

4. ENDPLATE OF THE SOLEUS MUSCLE FROM A RAT RECEIVING 0.1 mg/kg PHY ALONE. THE MUSCLE WAS REMOVED 1 Hr AFTER INJECTION OF PHY. THERE IS A SELECTIVE EFFECT ON Z LINES (ARROWS) WITHOUT ANY GROSS VACUOLIZATION OR MITOCHONDIAL SWELLING. Z LINES SHOW IRREGULARITIES AND DISSOLUTION.

CONCLUSIONS

(1) BOTH REVERSIBLE AND IRREVERSIBLE ANTI-ChE AGENTS, IN ADDITION TO THEIR ABILITY TO BLOCK ChE, DIRECTLY AFFECT THE NICOTINIC AChR. ON THE ENDPLATE CURRENTS, ChE INHIBITION CAUSES INCREASE IN THE PEAK AMPLITUDE AND PROLONGATION OF THE DECAY TIME CONSTANT. THE POSTSYNAPTIC EFFECTS OF THESE DRUGS RESULT FROM A BLOCKADE OF THE TRANSMITTER-ACTIVATED IONIC CHANNELS, AND/OR CHANNEL ACTIVATION OF THE NICOTINIC RECEPTOR THROUGH THEIR AGONIST ACTION. IN ADDITION, THESE AGONISTS ENHANCE RECEPTOR DESENSITIZATION.

(2) THE LACK OF ChE IN THE ISOLATED FROG MUSCLE FIBER MAKES THIS PREPARATION VERY USEFUL FOR STUDYING DIRECT EFFECTS OF ChE INHIBITORS. PATCH-CLAMP STUDIES PERFORMED IN THIS PREPARATION DISCLOSED ADDITIONAL FEATURES OF CHANNEL ACTIVATION IN THE PRESENCE OF THESE AGENTS.

(3) PHY EITHER ALONE OR IN THE PRESENCE OF ACh INDUCED CHANNEL OPENINGS WITH ALTERED CURRENT NOISE DURING THE OPEN STATE. INTERESTINGLY, THESE EVENTS WERE RECORDED AT PHY CONCENTRATIONS AS LOW AS 0.1 μM AND ALSO AS HIGH AS 600 μM, A CONCENTRATION AT WHICH ENDPLATE CURRENTS WERE BLOCKED.

(4) PHY, DFP, VX AND TABUN INDUCED AN INCREASE IN TRANSMITTER RELEASE IN THE LOCUST NEUROMUSCULAR JUNCTION WHICH GENERATED SPONTANEOUS EPPs AND APs. THESE EFFECTS WERE DEPENDENT ON THE EXTERNAL Ca^{2+} CONCENTRATION. A COMPLETE BLOCKADE BY TTX SUGGESTED A PRIMARY INTERFERENCE WITH THE INFLUX OF Na^{+} AT THE NERVE TERMINAL, RESULTING IN A TRANSIENT DEPOLARIZATION, AN INFLUX OF Ca^{2+}, AND, FINALLY, AN INCREASE IN TRANSMITTER RELEASE.

(5) THE COMPLEXITY OF THE INTERACTIONS OF BOTH REVERSIBLE AND IRREVERSIBLE ANTI-ChE AGENTS IN A NONCHLINERGIC SYSTEM IS REVEALED BY THE DISTINCT EFFECTS AT THE POSTSYNPATIC GLUTAMATE RECEPTORS OF THE LOCUST NEUROMUSCULAR JUNCTION.

(6) PHY IN COMBINATION WITH A GANGLIONIC BLOCKING DRUG GIVEN 30 MIN PRIOR TO VX ADMINISTRATION CAN OFFER 100% PROTECTION AGAINST 3.5 X THE LETHAL DOSE OF VX. MUSCLE FUNCTION EVALUATED THROUGH IN VIVO MUSCLE CONTRACTION MEASUREMENTS IN THE PRETREATED RATS SHOWED COMPLETE RECOVERY 24 Hr AFTER VX ADMINISTRATION.

(7) A PARTIAL PROTECTION OF BLOOD ChE AND MUSCLE AND BRAIN AChE (FROM IRREVERSIBLE PHOSPHORYLATION BY OP COMPOUNDS) BY A REVERSIBLE ChE INHIBITOR, DOES NOT EXPLAIN ALL OF THE RESULTS OBTAINED IN PROTECTION STUDIES. APPARENTLY IN ADDITION TO ChE INHIBITION DIRECT ACTIONS OF THESE AGENTS AT THE CHOLINERGIC AND NONCHOLINERGIC SYNAPSES MUST PLAY AN IMPORTANT ROLE IN THE PROTECTION MECHANISMS. SUCH A CONCLUSION IS SUPPORTED BY THE COMPLEX ACTIONS OF REVERSIBLE AND IRREVERSIBLE ChE INHIBITORS OBSERVED AT NICOTINIC AND GLUTAMATERGIC SYNAPSES AND THE STRIKING PROTECTION PROVIDED BY A DRUG WHICH IS DEVOID OF ANTI-ChE ACTIVITY.

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CHANGES IN ACETYLCHOLINE RELEASE AFTER CHRONIC EXPOSURE TO ANTICHOLINESTERASES

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BACKGROUND

The search for neurochemical mechanisms in tolerance to anticholinesterase compounds (antiChEs) has focused on downregulation of postsynaptic muscarinic receptors (mAChRs) in the CNS during chronic exposure. Down-regulation is associated with the development of subsensitivity as behavioral and physiological variables return toward pre-exposure states.

AIM

The present experiments were designed to study the possible involvement of muscarinic autoreceptors. Such receptors function in the control of ACh release from presynaptic nerve terminals and might be involved as homeostatic processes in the development of tolerance (Russell et al, 1981).

METHOD

There being no selective ligand for such receptors, the rat myenteric plexus preparation was used as a peripheral model. Groups of rats were injected S.C. acutely or chronically with the antiChEs, DFP or soman, or with their respective vehicles. Animals were sacrificed at various times after injection. The longitudinal muscle of the ileum with associated myenteric plexus was teased from its underlying circular layer (Paton and Vizi, 1969).

Strips were mounted on platinum electrodes and the preparation immersed in tubes containing 3 ml Tyrode solution with 30μM eserine (See Figure 1). Thirty min treatment periods included: 10 min resting, 10 min stimulation, 10 min recovery (Kilbinger, 1977). During some of these periods strips were exposed to atropine at concentrations of 10^{-9} to 10^{-5}M. Stimulation was at 3Hz for 1 msec with a current of 200 mA. Contents of each tube was analyzed by GCMS for ACh and Ch (Jenden et al, 1973). Finally, strips were similarly analyzed and brain tissue assayed for AChE activity.

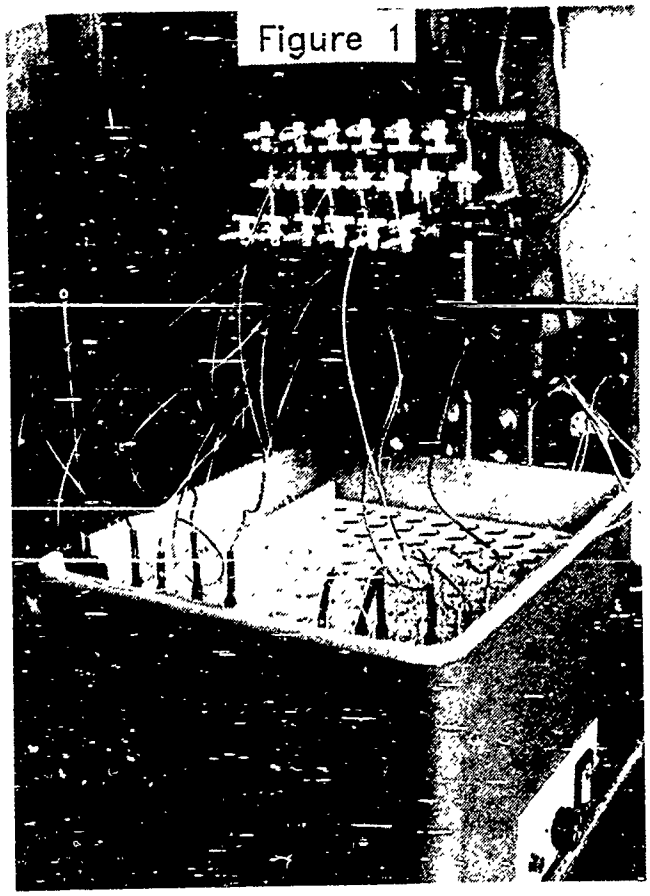


Figure 1

RESULTS

1. In all DFP and soman treated subjects AChE activity was significantly reduced in brain and plexus.
2. ACh release from animals sacrificed at various times after acute injection did not differ from control values.
3. Resting release of ACh after the chronic DFP and soman regimen was not affected.
4. Evoked release (stimulated−resting) after the chronic regimen with both DFP and soman was signigicantly greater, as shown in Figures 2 and 3.
5. Evoked release under both conditions showed a dose−dependent disinhibition when tissues were stimulated in atropine solutions.

Figure 2

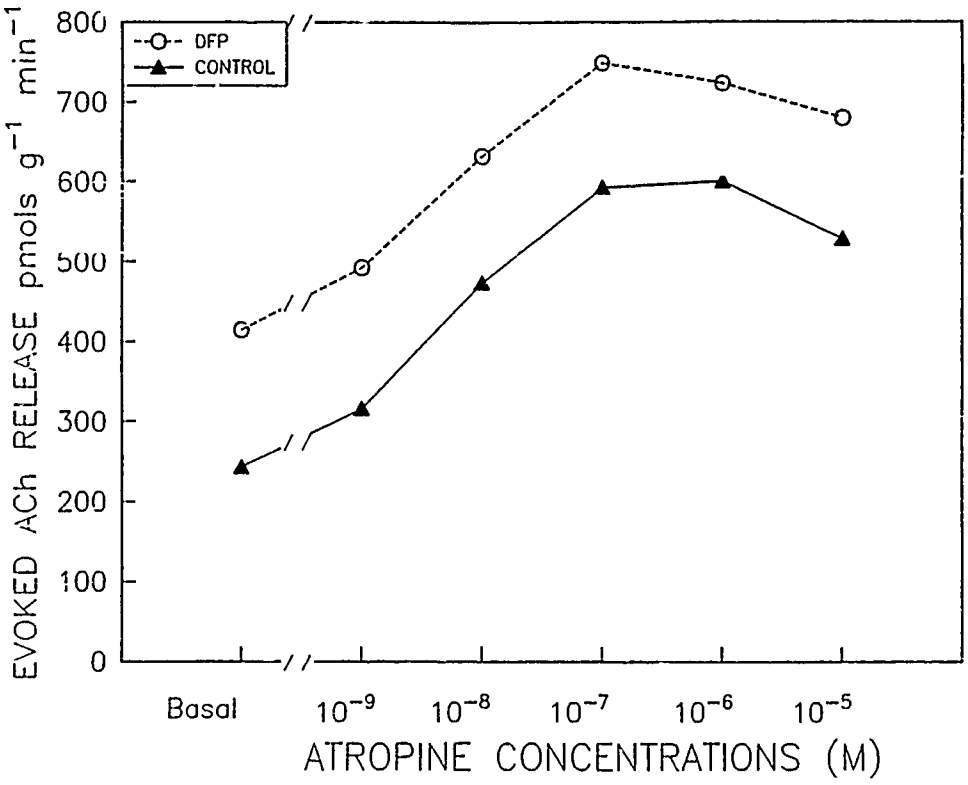
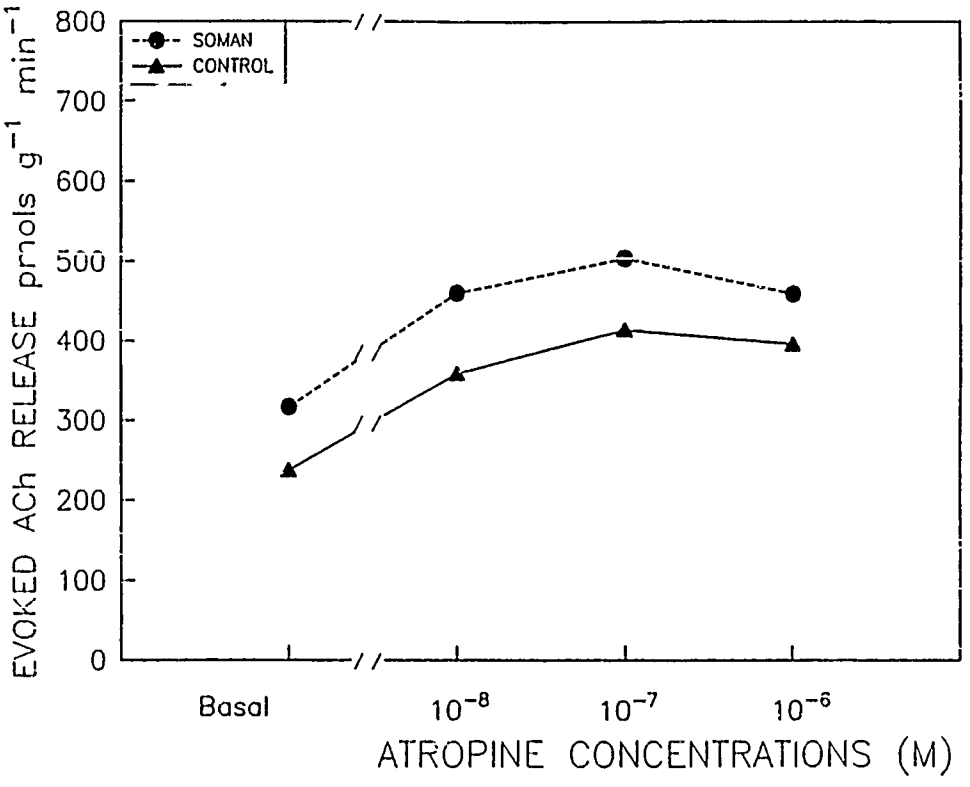


Figure 3



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CONCLUSIONS

The present results support an alternative role for presynaptic mAChRs than that which we had hypothesized: following decreases in AChE activity induced by antiChEs, down-regulation of the autoreceptors leads to greater release of ACh which, in turn, modulates the downregulation of postsynaptic mAChRs. The downregulation takes some 4 days to stabilize and continues as long as the primary stimulus, decreased AChE activity, persists. Such a series of events could occur concomitantly with recovery of behavioral and physiological variables to pretreatment states, i.e. development of tolerance.

EFFECTS OF DFP, SOMAN AND SARIN ON CATECHOLAMINE AND SEROTONIN LEVELS IN SELECTED BRAIN REGIONS OF MICE

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ABSTRACT

DFP (1 AND 3 MG/KG), SOMAN (12.5, 25 AND 37.5 UG/KG) AND SARIN (60, 80 AND 100 UG/KG) WERE GIVEN I.V. TO MICE WHICH WERE SACRIFICED 10 MIN AFTER INJECTION. SALINE-INJECTED ANIMALS SERVED AS CONTROLS. LEVELS OF NOREPINEPHRINE (NE), DOPAMINE (DA), 5-HYDROXYINDOLEACETIC ACID (5-HIAA) AND SEROTONIN (5-HT) WERE DETERMINED IN SIX BRAIN REGIONS (CORTEX, MEDULLA, MIDBRAIN, HIPPOCAMPUS, CORPUS STRIATUM AND CEREBELLUM). ONLY THE HIGHEST DOSE (3 MG/KG) OF DFP LOWERED SIGNIFICANTLY ($P = < 0.01$) THE LEVEL OF DA IN THE CORPUS STRIATUM, WHILE NO IMPORTANT DIFFERENCES WERE FOUND WITH EITHER DOSE IN THE OTHER 5 BRAIN REGIONS. SOMAN (37.5 UG/KG) INCREASED ($P = < 0.01$) THE LEVEL OF DA IN THE MEDULLA WHILE OTHER DOSES (12.5 AND 25 UG/KG) WERE WITHOUT EFFECT. LEVELS OF 5-HT WERE INCREASED ($P = 0.05$) IN THE MEDULLA AFTER SOMAN TREATMENT (37.5 UG/KG) WHILE LEVELS OF NE AND 5-HIAA WERE UNCHANGED AFTER ALL THREE DOSES. BOTH DA AND 5-HT LEVELS WERE INCREASED ($P = < 0.01$) IN MOUSE CORTEX AFTER SOMAN TREATMENT (37.5 UG/KG) BUT LEVELS OF NE AND 5-HIAA WERE UNCHANGED WITH ALL DOSES. NO STATISTICAL DIFFERENCES IN LEVELS OF NE, DA, 5-HIAA AND 5-HT IN THE SIX BRAIN REGIONS WERE FOUND IN SARIN-TREATED ANIMALS IRRESPECTIVE OF DOSE. OUR FINDINGS INDICATE THAT THESE CHOLINESTERASE INHIBITORS HAVE A MINIMAL DIRECT EFFECT ON THE LEVELS OF TRANSMITTERS IN THE CATECHOLAMINERGIC AND SEROTONIN - MEDIATED SYSTEMS.

INTRODUCTION

THE GOAL OF OUR PROJECT IS TO DETERMINE WHETHER THE INTRAVENOUS INJECTION OF VARIABLE DOSES OF IRREVERSIBLE ACETYLCHOLINESTERASE INHIBITORS HAVE A DIRECT EFFECT ON NEUROTRANSMITTER SYSTEMS OTHER THAN THE CHOLINERGIC SYSTEM. EXISTING EVIDENCE SUGGESTS THAT THE TOXIC EFFECTS, ESPECIALLY THOSE FOLLOWING LONG TERM EXPOSURE, ARE DUE TO MORE THAN ACETYLCHOLINESTERASE INHIBITION. IN THE PRESENT STUDY, A TIME PERIOD OF 10 MINUTES AFTER INTRAVENOUS ADMINISTRATION TO MICE, WAS USED TO DETERMINE THE ACUTE EFFECTS OF THESE COMPOUNDS ON BEHAVIOR AND CHANGES THAT THEY CAUSE IN LEVELS OF BIOGENIC AMINES IN BRAIN REGIONS.

ACKNOWLEDGEMENTS

THE AUTHORS ACKNOWLEDGE GRATEFULLY THE SUPPORT OF THE U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT CONTRACT 17-82-C174.

Extraction of Biogenic Amines and Metabolites from Brain Tissue of Organophosphate-Treated Mice

Inject agent via tail vein

↓

Decapitate animal after 10 min and remove brain

↓

Dissect brain into six areas (cerebellum, medulla, midbrain, hippocampus, corpus striatum, cortex)

↓

Add 0.9 ml 0.4 M perchloric acid + internal standard
Homogenize with polytron

↓

Centrifuge at 4^{0} C and 8000 rpm

↓

Supernatant adjusted to pH5
(10 M potassium acetate)

↓

Centrifuge to remove $KClO_4$

↓

Inject supernatant into HPLC system

Liquid Chromatographic Operating Conditions for Assay of Biogenic Amines and Metabolites in Brain Tissue

Liquid Chromatograph: LC304T (Bioanalytical Systems, Inc.) used with a Waters Wisp 710B Sample Processer and a Waters 730 Data Module.

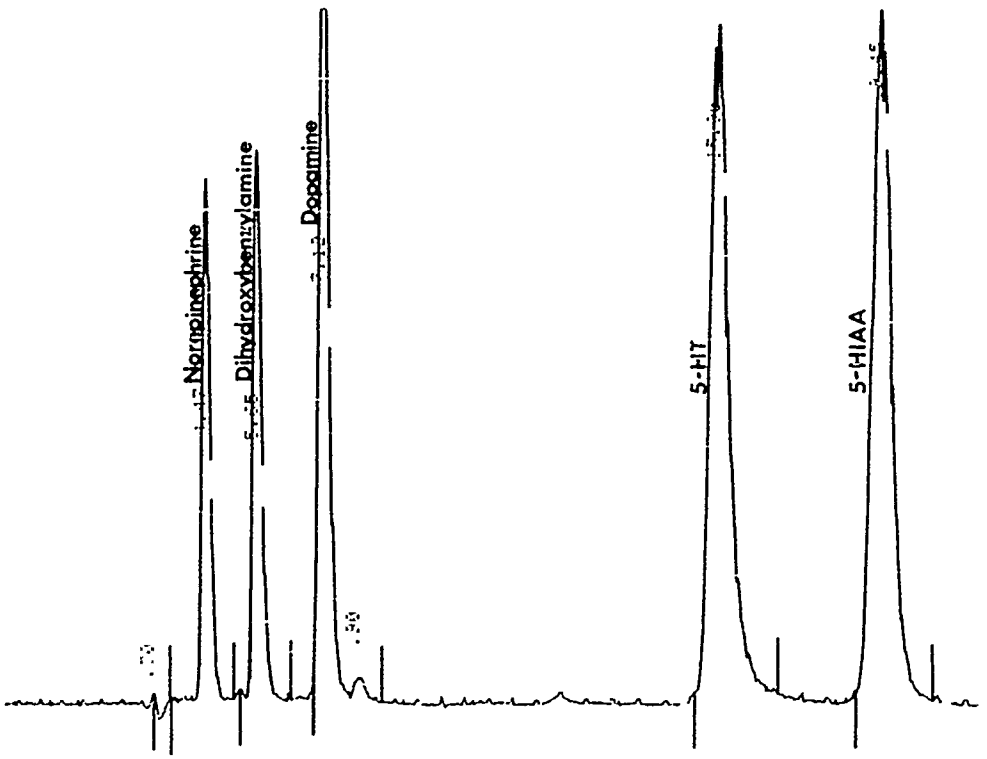
Mobile Phase: 0.15 M monochloroacetic acid (pH 3.0) containing 2 mM Na_2 EDTA and 25-30 mg/L hexane sulfonic acid: 15% CH_3CN (v/v).

Stationary Phase: Biophase ODS 5 u Column (4 x 250 mm) at 30^{0} C.

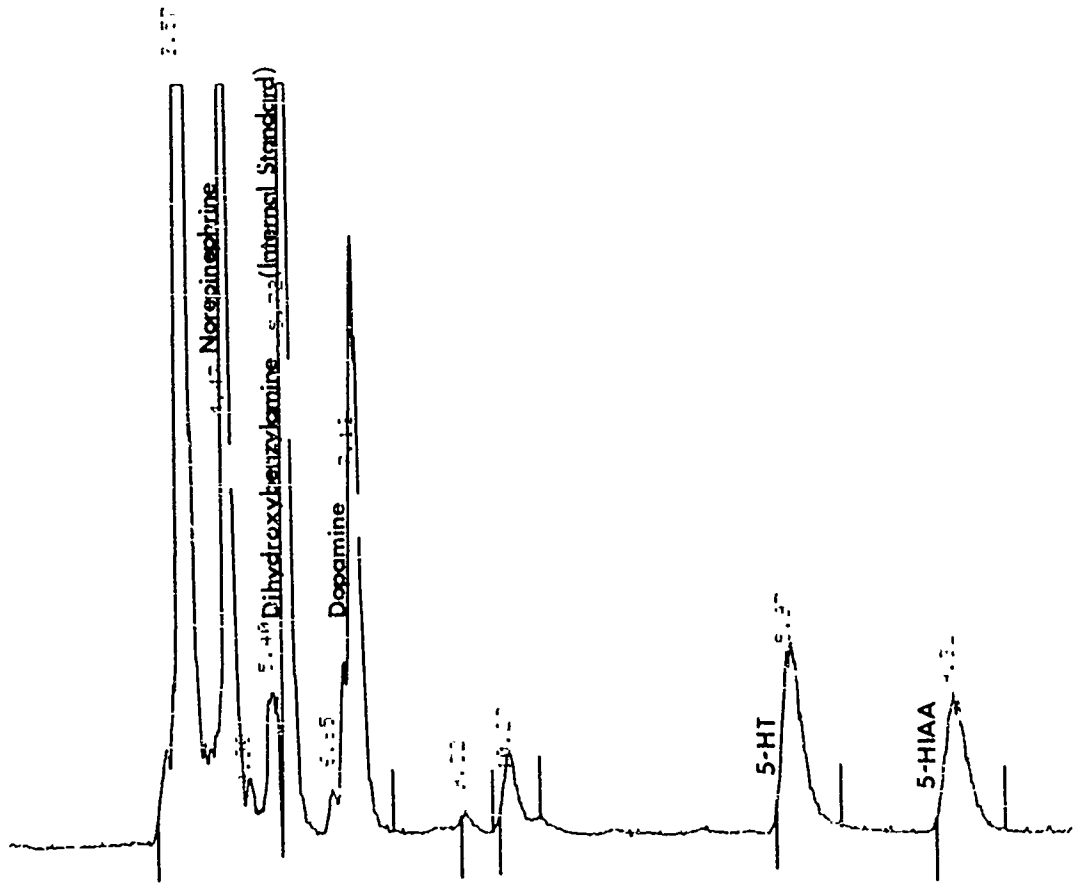
Detector: LC-4B electronic controller and LC-17 oxidative flow cell with a TL-3/cP-o working electrode. Applied potential + 550 mV vs Ag/AgCl.

Flow Rate: 0.9 ml/min

Amount Injected: 100 ul



Standards



Supernatant from Brain Homogenate

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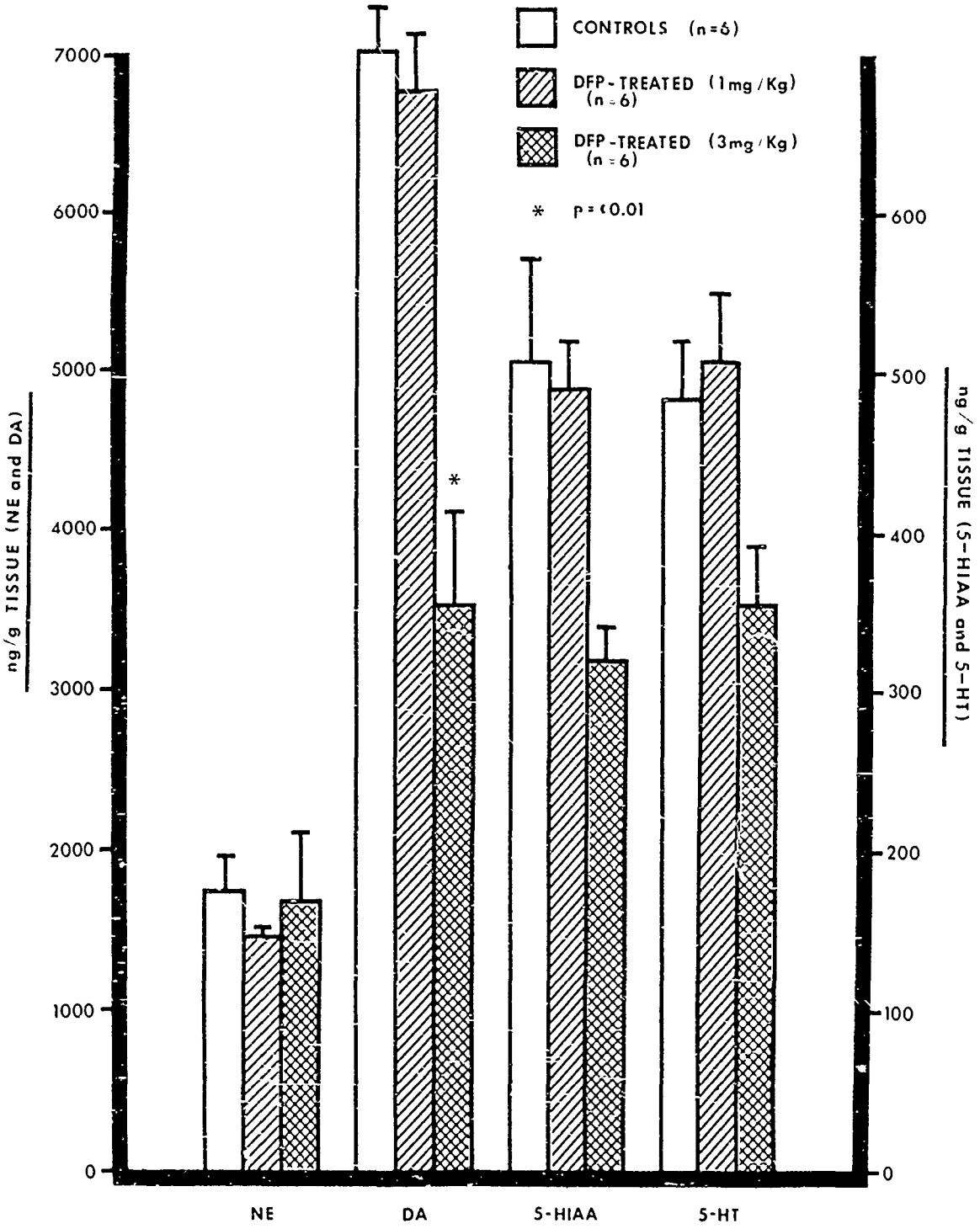
Effect of DFP or Levels of NE, DA, 5-HIAA and 5-HT in Mouse Corpus Striatum.



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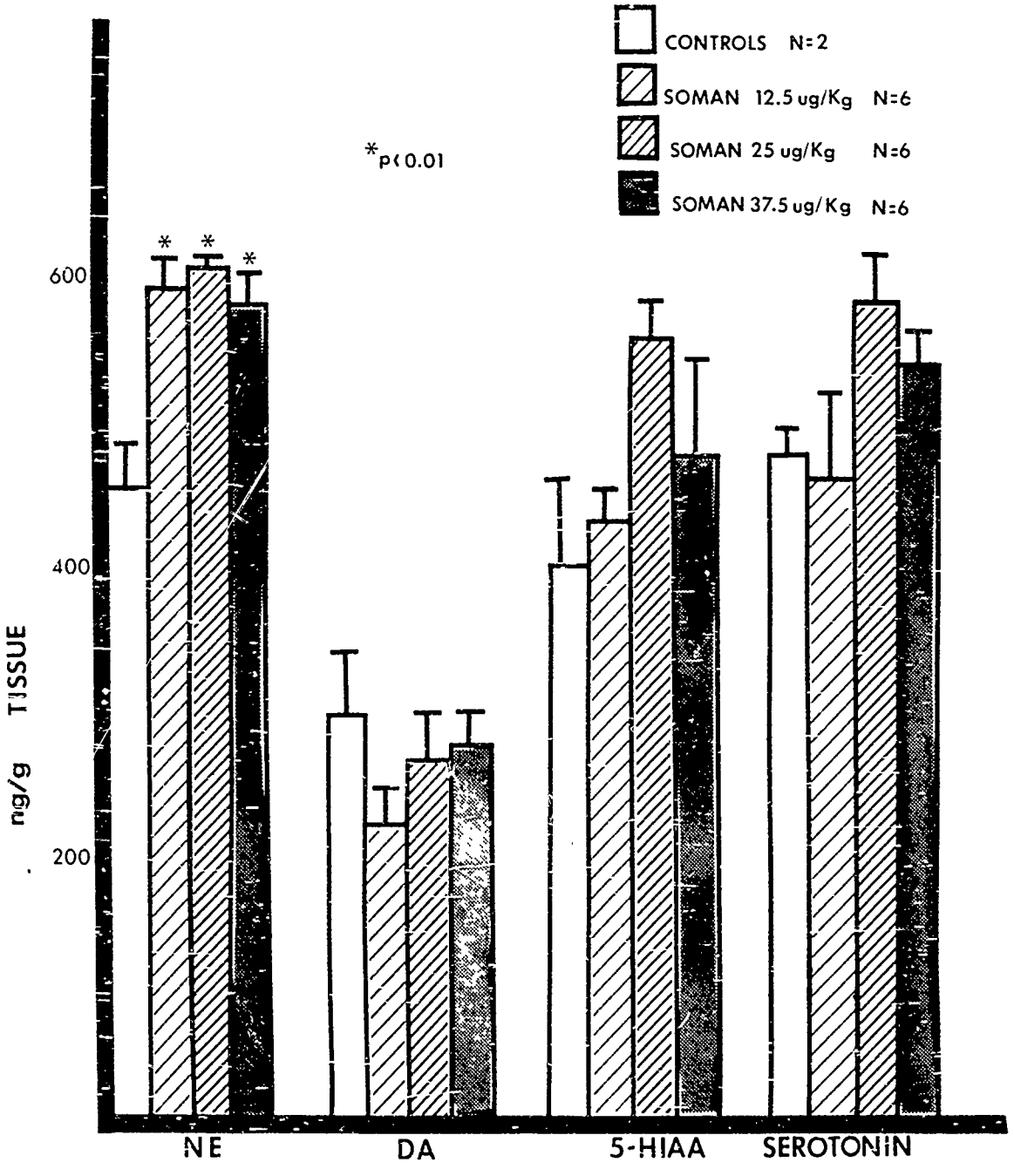
Effect of Soman on Levels of Norepinephrine (NE), Dopamine (DA), 5-Hydroxyindolacetic Acid (5-HIAA), and Serotonin in the Mouse Midbrain.



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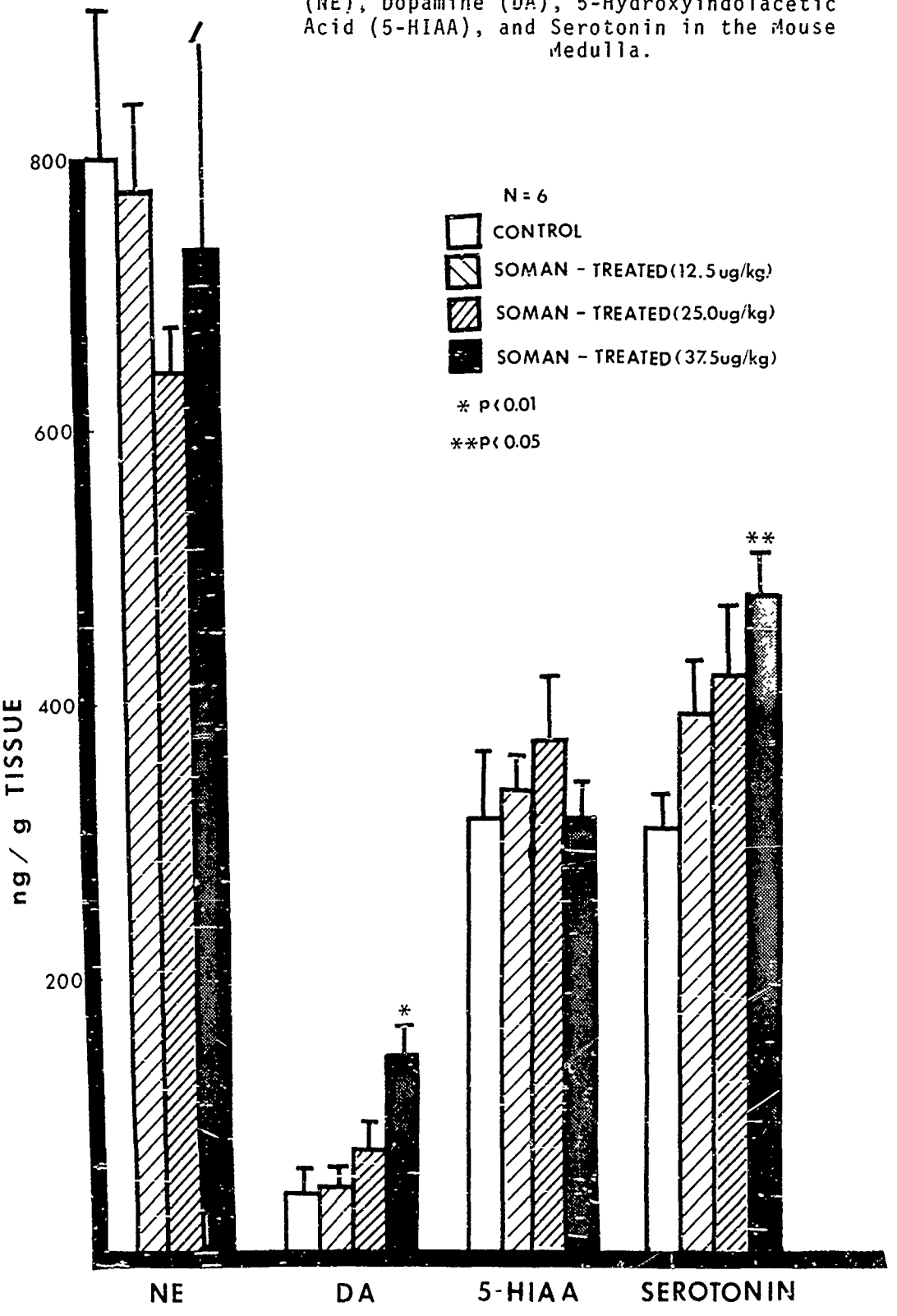
Effect of Soman on Levels of Norepinephrine (NE), Dopamine (DA), 5-Hydroxyindolacetic Acid (5-HIAA), and Serotonin in the Mouse Medulla.



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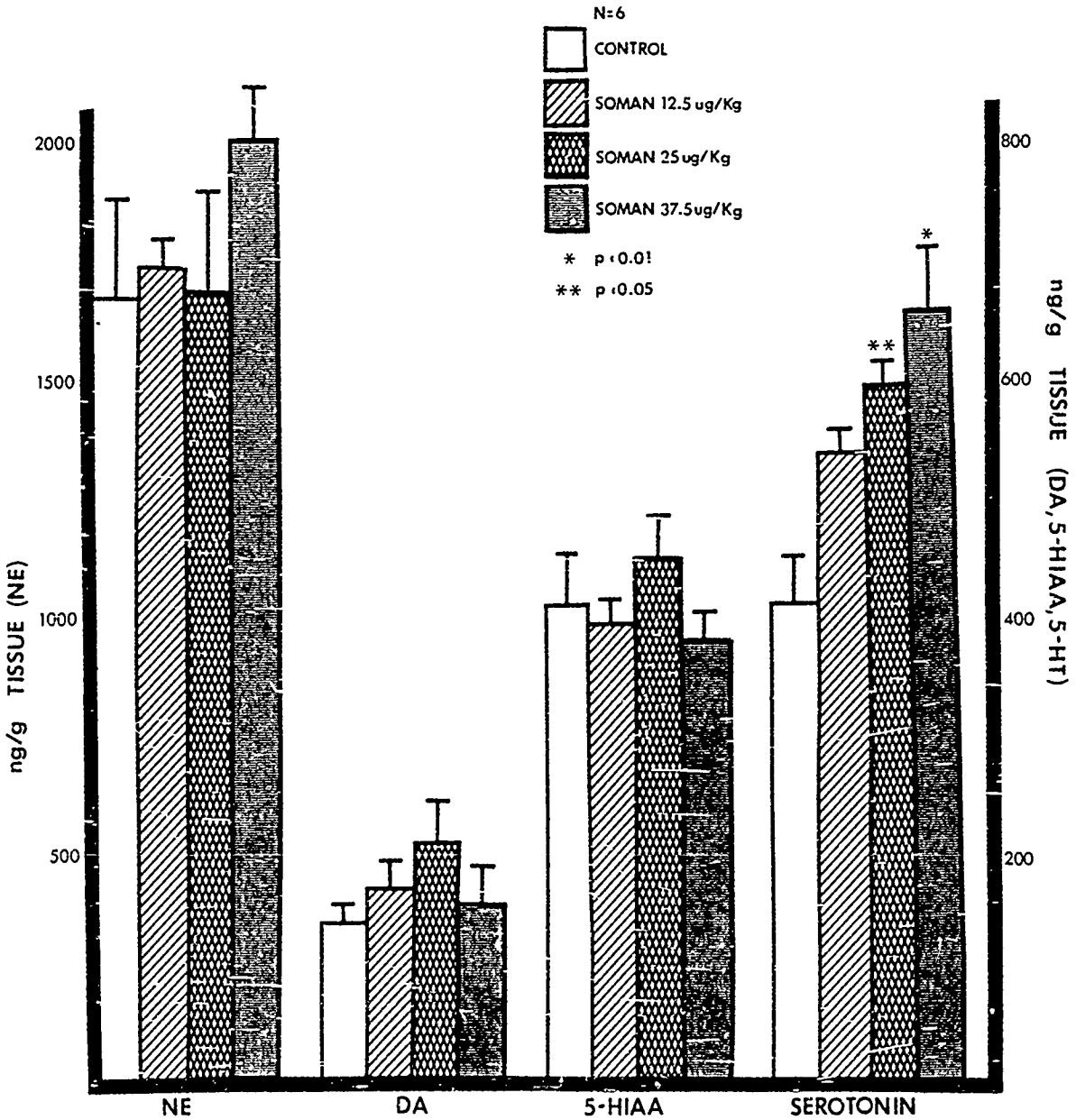
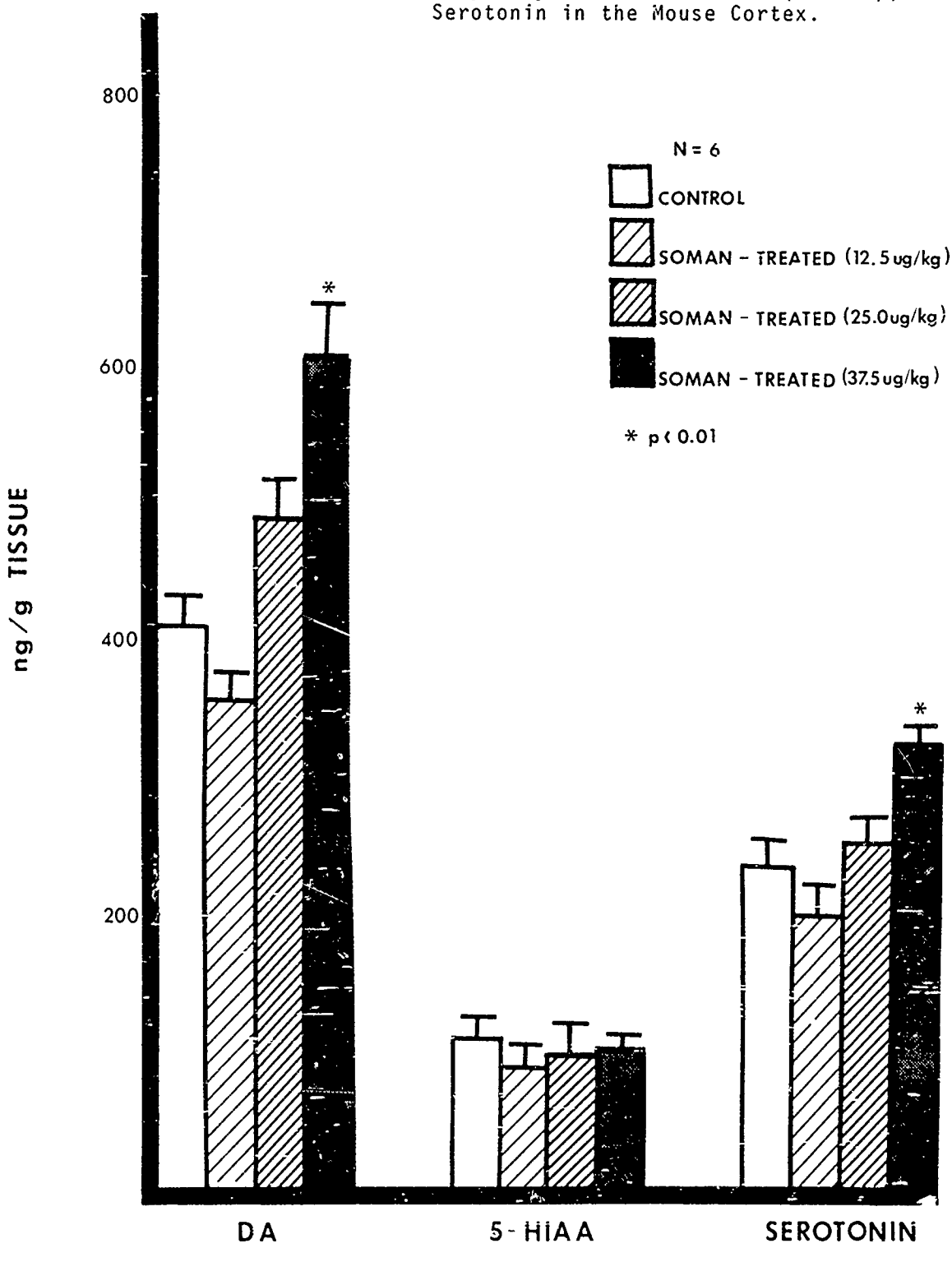
Effect of Soman on Levels of Norepinephrin (NE), Dopamine (DA), 5-Hydroxyindolacetic Acid (5-HIAA), and Serotonin in the Mouse Hippocampus.



Effect of Soman on Levels of Dopamine (DA), 5-Hydroxyindoleacetic Acid (5-HIAA), and Serotonin in the Mouse Cortex.



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SUMMARY AND CONCLUSIONS

1. DFP FAILED TO ALTER THE LEVELS OF NE, 5-HIAA AND 5-HT IN SIX BRAIN REGIONS OF MICE WHILE THE DA LEVELS WERE LOWERED SIGNIFICANTLY IN THE CORPUS STRIATUM (WITH 3 MG/KG DFP) WITH NO CHANGES IN LEVELS OF DA IN THE CEREBELLUM, MEDULLA, MIDBRAIN, HIPPOCAMPUS AND CORTEX.

2. SOMAN RAISED SIGNIFICANTLY THE LEVELS OF DA AND 5-HT IN THE MOUSE MEDULLA WHILE LEVELS OF NE WERE ELEVATED SIGNIFICANTLY IN THE MIDBRAIN.

3. SOMAN ELEVATED SIGNIFICANTLY THE LEVELS OF DA AND 5-HT IN THE MOUSE CORTEX.

4. WORK IS IN PROGRESS TO CONFIRM AND EXTEND THE ABOVE OBSERVATIONS.

5. THESE FINDINGS SUGGEST THAT ACUTE TREATMENT WITH CHOLINESTERASE INHIBITORS HAS A MINIMAL EFFECT ON CATECHOLAMINE AND SEROTONIN LEVELS.

CORRELATION OF THE BEHAVIORAL EFFECTS OF ORGANOPHOSPHATE CHOLINESTERASE INHIBITORS AND THEIR EFFECTS ON MOUSE BRAIN NEUROCHEMISTRY

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ABSTRACT

THE INTRAVENOUS LD-50 OF SOMAN, SARIN AND TABUN HAVE BEEN FOUND TO BE 42(35-50), 109(101-118) AND APPROXIMATELY 250 UG/KG, RESPECTIVELY. WE HAVE STUDIED THE EFFECTS OF THESE COMPOUNDS ON SPONTANEOUS ACTIVITY AND BODY TEMPERATURE AT DOSES WHICH PRODUCED COMPARABLE INHIBITION OF BRAIN CHOLINESTERASE. ALL THREE COMPOUNDS CAUSED A DECREASE IN SPONTANEOUS ACTIVITY AND BODY TEMPERATURE ALTHOUGH A GOOD DOSE RESPONSE RELATIONSHIP WAS NOT ALWAYS OBSERVED. INHIBITION OF BRAIN ENZYME GENERALLY LASTED LONGER THAN THE DECREASE IN SPONTANEOUS ACTIVITY OR BODY TEMPERATURE SUGGESTING THAT THE BRAIN COULD COMPENSATE FOR THE ENZYME INHIBITION OR ANOTHER NEUROCHEMICAL CHANGE WAS RESPONSIBLE FOR THE ALTERATIONS IN BEHAVIOR. DFP AT A DOSE OF 2 MG/KG SIGNIFICANTLY BLOCKED CHOLINESTERASE ACTIVITY AND CAUSED AN INCREASE IN MOUSE WHOLE BRAIN LEVELS OF ACETYLCHOLINE. WHEN BRAIN REGION STUDIES WERE COMPLETED IT WAS OBVIOUS THAT THE INCREASE IN ACETYLCHOLINE LEVEL WAS IN THE MIDBRAIN AND HIPPOCAMPUS REGIONS. DFP AT DOSES OF 1 AND 2 MG/KG, SOMAN AT 12.5, 25 AND 37.5 UG/KG AND SARIN AT 60, 80 AND 100 UG/KG DID NOT ALTER IN A CONSISTENT DOSE RESPONSIVE FASHION BRAIN LEVELS OR TURNOVER RATE OF NOREPINEPHRINE, DOPAMINE OR SEROTONIN. THE DIFFERENT NEUROCHEMICAL PROFILES OF ACTIVITY FOR THESE COMPOUNDS SUGGESTS THAT, ALTHOUGH THESE CHANGES DO NOT ACCOUNT FOR THE BEHAVIORAL CHANGES OBSERVED, THERE ARE DIFFERENCES AMONG THE EFFECTS OF THE IRREVERSIBLE CHOLINESTERASE INHIBITORS ON THESE OTHER BRAIN NEUROCHEMICAL SYSTEMS. THESE DIFFERENCES WARRENT FURTHER INVESTIGATION.

THIS WORK SUPPORTED IN PART BY THE U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND UNDER CONTRACT DAMD-17-82-C-2174.

DISCUSSION

Time course effect of organophosphates on AChE activity. The time of AChE inhibition in mouse brain after soman 37.5, sarin 80 and tabun 200 ug/kg administration are presented in tables 1, 2 and 3, respectively. The maximum inhibition was noticed upto 24 hr except sarin where partial recovery was seen. Gradual recovery of AChE activity was found after 4, 7 and 14 days but the enzyme did not recover completely even after 14 days.

Dose-related effect of organophosphates on AChE activity in whole mouse brain. The effect of various doses of soman, sarin and tabun are shown in tables 4, 5 and 6, respectively. Dose-dependent effects were observed by each organophosphate. The highest doses of soman (37.5 ug/kg), sarin (100 ug/kg) and tabun (200 ug/kg) produced similar behavioral effects but inhibited brain AChE to a different extent; 70, 88 and 72%, respectively.

Dose-related effect of organophosphates on AChE activity in mouse brain areas. The same doses of soman, sarin and tabun were used which were used for inhibition of AChE activity in whole mouse brain. The data are shown in tables 7, 8 and 9, respectively. All three organophosphates showed good dose-related effects in every brain. Each brain area was almost equally inhibited with all three organophosphates except corpus-striatum which showed less inhibition following soman.

FIGURE 3
SPONTANEOUS ACTIVITY IN MICE AFTER IV TREATMENT WITH SALINE (●) OR 25 μg/kg SOMAN (▲).
ACTIVITY MEASURED 0-10 MIN AFTER PLACEMENT INTO CHAMBERS

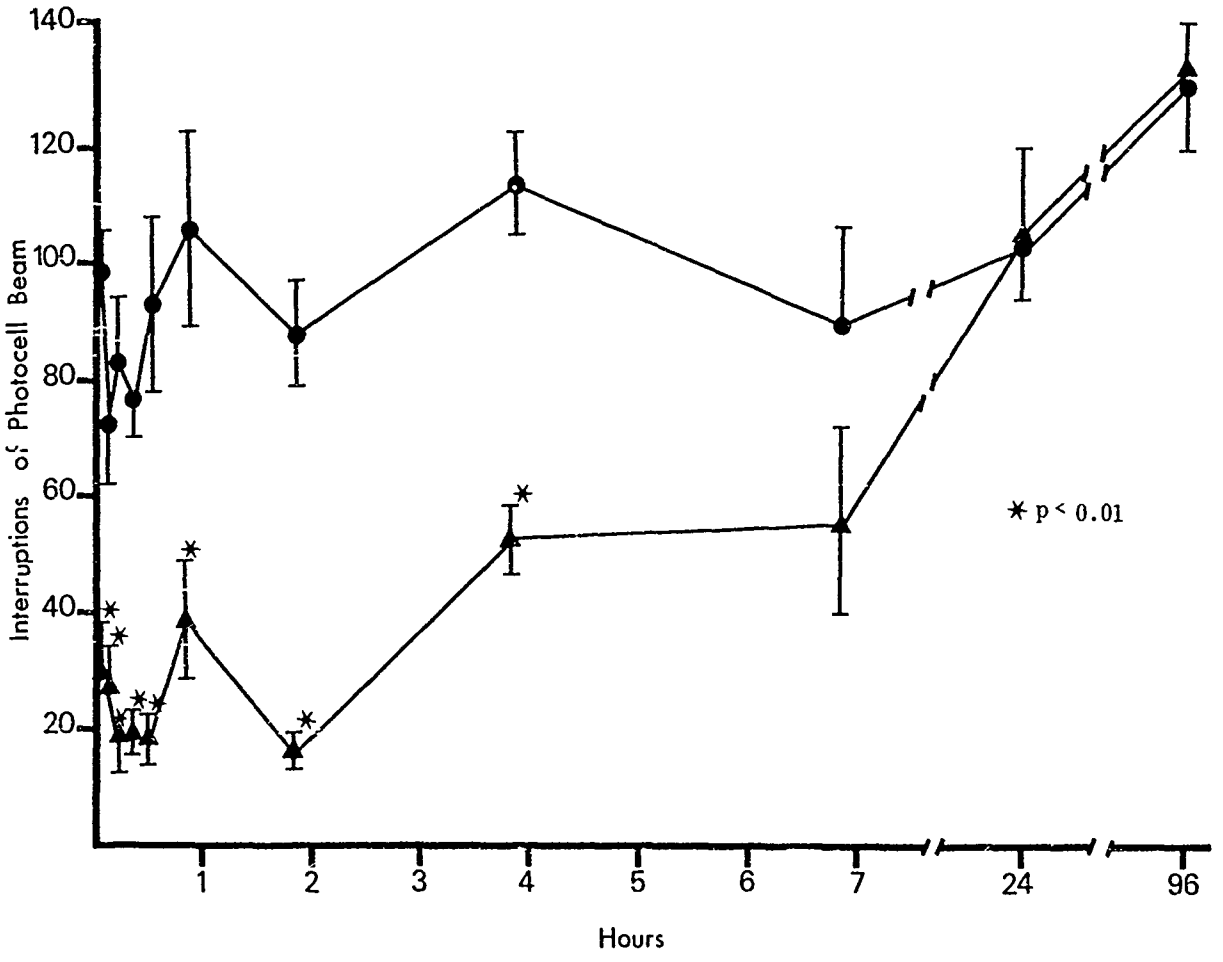


TABLE 1

TIME COURSE EFFECT OF SOMAN (I.V.) ON ACHE ACTIVITY
IN MOUSE BRAIN

	PERCENT INHIBITION	
TIME	25 UG/KG	37.5 UG/KG
1 MIN	44 ± 3.3	----
4 HRS	44.1 ± 3.3	72.5 ± 3.6
24 HRS	38.1 ± 4.1	70.1 ± 4.25
4 DAYS	----	51.3 ± 1.6
7 DAYS	----	45.0 ± 2.0
14 DAYS	----	25.5 ± 2.6

TIME COURSE OF SOMAN-INDUCED HYPOTHERMIA IN MICE

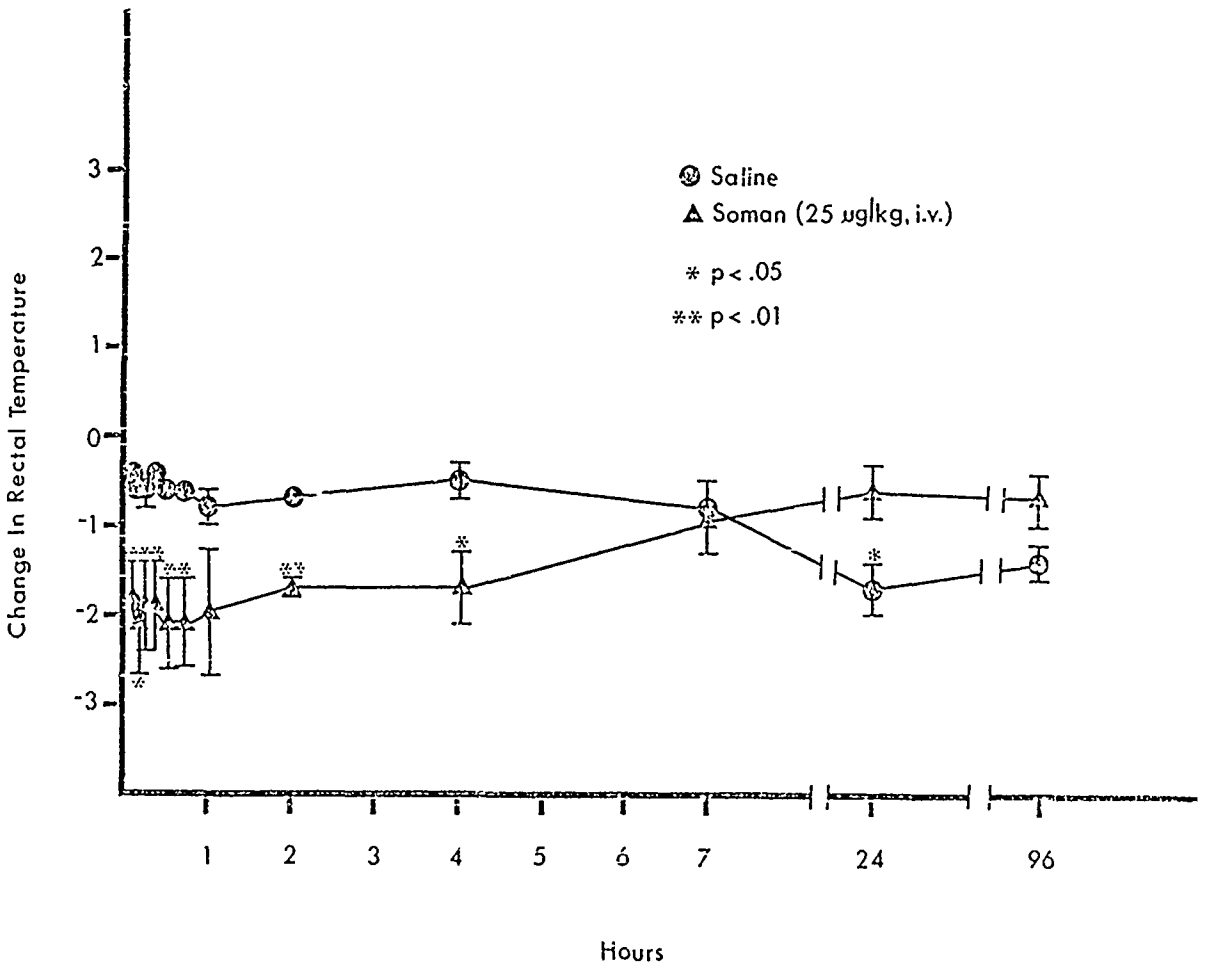


TABLE 4

DOSE-RESPONSE EFFECT OF SOMAN ON ACETYLCHOLINESTERASE IN MOUSE BRAIN

DOSE (UG/KG, I.V.)	% INHIBITION
12.5	10.2 ± 2.5
25.0	44.0 ± 1.9
37.5	70.0 ± 1.6

ED50 = 27.3 (16.7 ± 44.5).

MICE WERE SACRIFICED AFTER 10' MIN POST INJECTION.

TIME COURSE OF SPONTANEOUS ACTIVITY FOLLOWING ADMINISTRATION OF SARIN

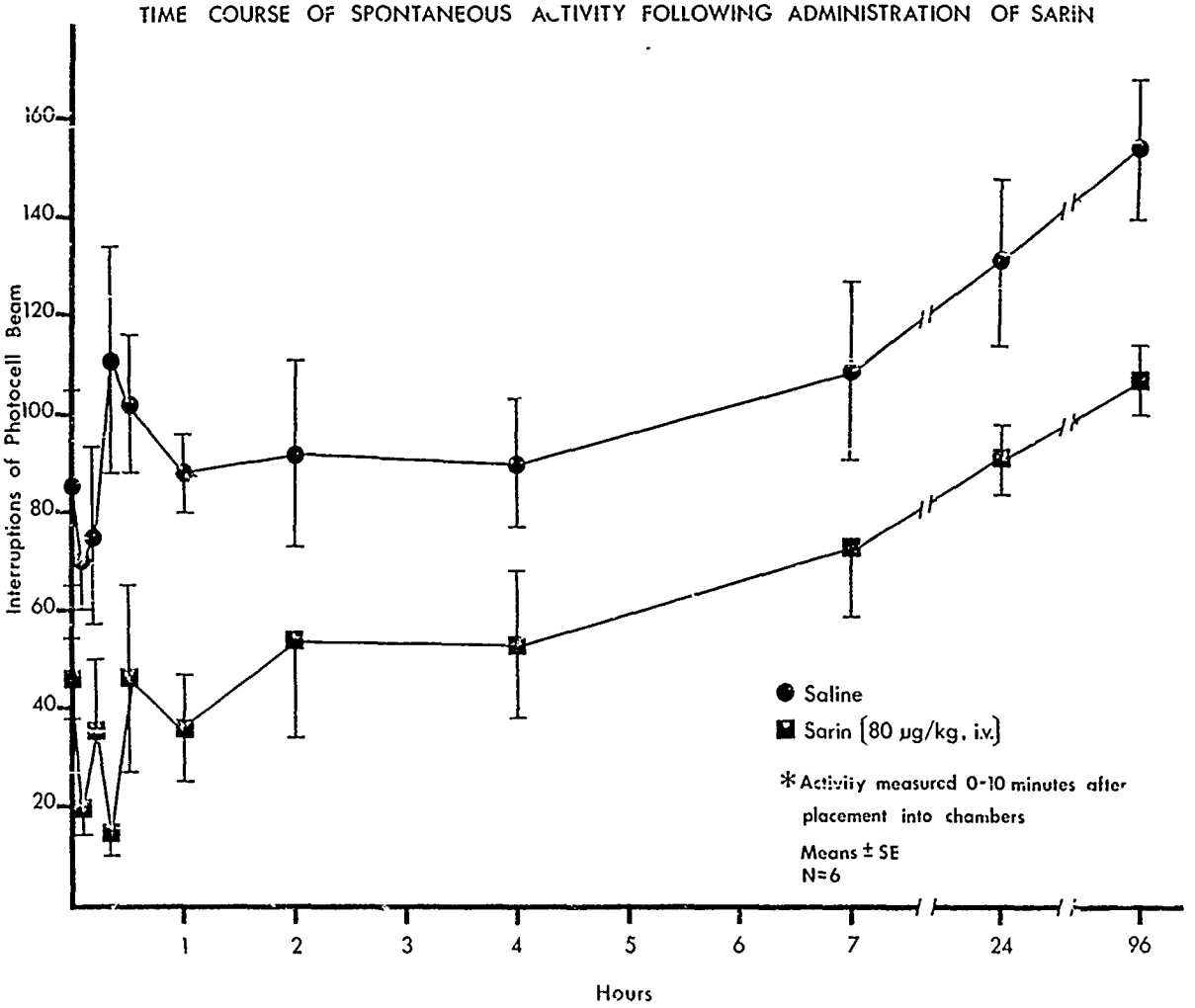


TABLE 2

TIME COURSE EFFECT OF SARIN (80 UG/KG, I.V.) ON ACHE ACTIVITY IN MOUSE BRAIN

TIME	PERCENT INHIBITION
4 HRS	83.9 ± 1.9 (N = 4)
24 HRS	66.6 ± 5.7
4 DAYS	28.7 ± 4.84
7 DAYS	31.3 ± 4.5 (N = 5)
14 DAYS	11.2 ± 4.5

TIME COURSE OF HYPOTHERMIA FOLLOWING ADMINISTRATION OF SARIN

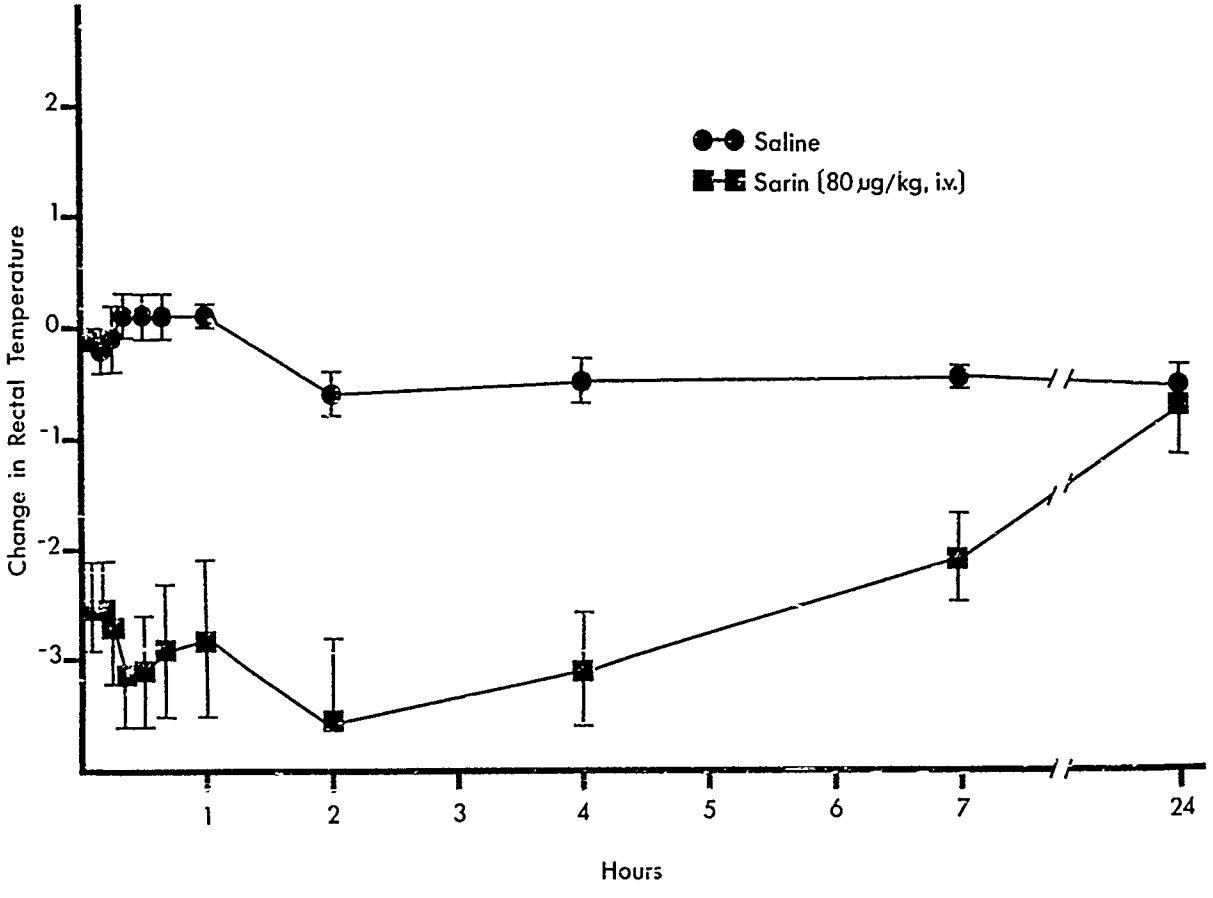


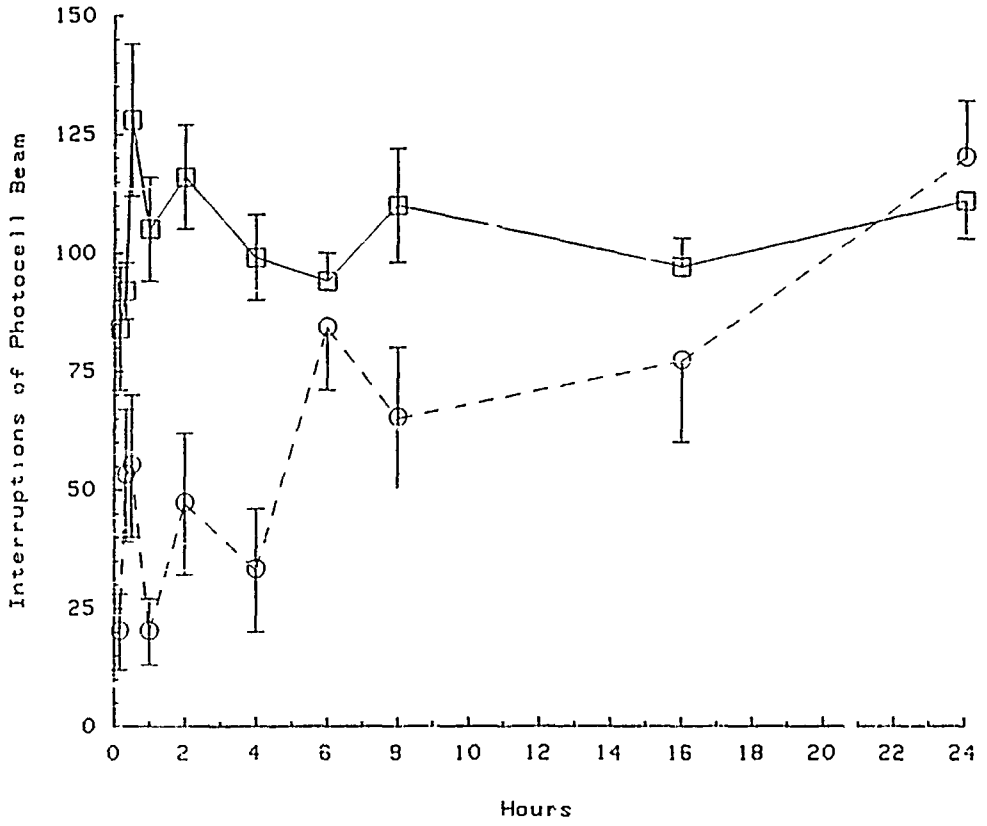
TABLE 5

DOSE-RESPONSE EFFECT OF SARIN ON ACETYLCHOLINESTERASE IN MOUSE BRAIN

DOSE (UG/KG)	% INHIBITION
20	25.0 ± 1.8
40	51.9 ± 5.2
60	76.0 ± 1.7
80	84.3 ± 2.4
100	88.4 ± 2.9 (N = 3) (6 OUT OF 9 MICE DIED)

ED50 = 40.8 (17.1 ± 97.1) UG/KG.

TABUN


Interruptions of Photocell Beam
150
125
100
75
50
25
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours

TABUN

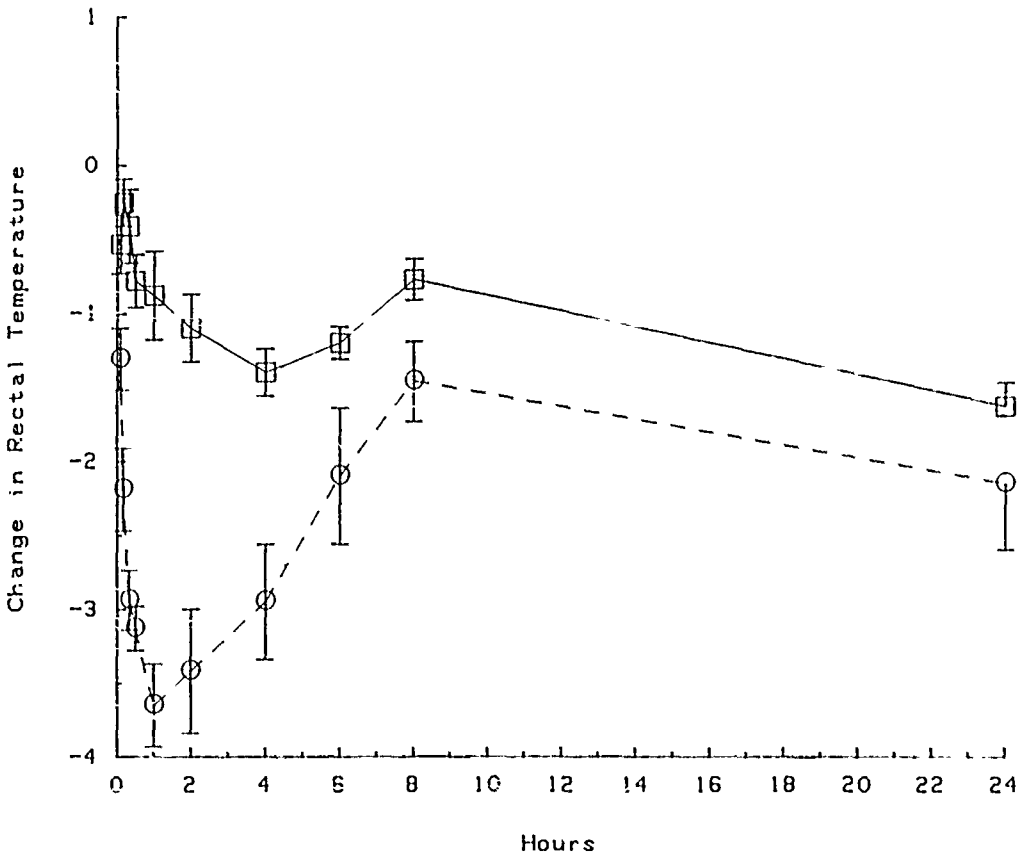

Change in Rectal Temperature
1
0
-1
-2
-3
-4
0
2
4
6
8
10
12
14
16
18
20
22
24
Hours

TABLE 3

TIME COURSE EFFECT OF TABUN (200 UG/KG, I.V.) ON ACHE ACTIVITY IN MOUSE BRAIN

TIME	% INHIBITION
1 MIN	48.9 ± 4.1
4 HRS	52.0 ± 12.7
24 HRS	50.0 ± 9.5
4 DAYS	29.5 ± 3.8
7 DAYS	20.9 ± 5.1
14 DAYS	21.3 ± 1.7

THIS EXPERIMENT WAS DONE AFTER THREE WEEKS OF TABUN ARRIVAL.

TABLE 6

DOSE RESPONSE EFFECT OF TABUN ON ACETYLCHOLINESTERASE IN MOUSE BRAIN

DOSE UG/KG	% INHIBITION
175	56.9 ± 6.9
200	69.2 ± 5.1
225	71.6 ± 5.6

ED50 = 145.9 (66.8 - 318.6) UG/KG.

ATROPINE-INSENSITIVE NEUROTOXICITY OF OXOTREMORINE

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Oxotremorine is a highly selective ligand of the muscarinic cholinergic receptor and is widely used as a tool in investigations of muscarinic function. Oxotremorine produces signs of parasympathetic activation and produces central nervous system actions which are in accord with the idea that the predominant pharmacology of oxotremorine is based upon binding to muscarinic receptors. However, studies with squirrel monkeys have led us to suspect that oxotremorine may have neurotoxic actions which are not mediated through muscarinic receptors. This report documents that relatively high doses of oxotremorine produce neurotoxic actions which are unresponsive to muscarinic blockade.

The ability of atropine sulfate to prevent peripheral parasympathetic actions (salivation, lacrimation, urination, defecation), tremor, convulsions, and death produced by oxotremorine (0.5-20 mg/kg, i.p.) was studied in male Sprague-Dawley rats. Peripheral parasympathetic activation by oxotremorine was dose- and time-dependent and was completely blocked by a 15 min pre-treatment with atropine (10 mg/kg, i.p.). The percentage of animals exhibiting tremor, convulsions, and death was also dose- and time-dependent. Although atropine (10 mg/kg) prevented tremor, convulsions, and death at doses of 5 mg/kg oxotremorine and below, at higher doses of oxotremorine (7.5 - 20 mg/kg) convulsions were not affected, and tremor and death could not be completely prevented by 40 mg/kg atropine. Under these conditions, peripheral parasympathetic effects of oxotremorine were absent. Atropine alone was without observable effect on these measures. The LD-50 for oxotremorine was 8.3 mg/kg. Cyanosis preceded death. The lethality function was shifted two-fold to the right by 40 mg/kg atropine. Although death only occurred in convulsing animals, lethal effects of oxotremorine could be differentiated from its convulsive effects since convulsions were not affected by atropine.

The inability of atropine to prevent oxotremorine-induced neurotoxicity was not due to the use of insufficient concentrations of atropine, time parameters, or routes of administration. Doses of atropine from 5 to 160 mg/kg, i.p., or 40 mg/kg, s.c. were also ineffective; administration of atropine at 15, 30, 45, or 60 min prior to oxotremorine or atropine given during convulsive episodes was also inadequate to protect or reverse neurotoxic actions of high oxotremorine doses. The neurotoxicity of relatively high doses of oxotremorine appears to be unrelated to actions of oxotremorine at muscarinic receptors since, in addition to atropine, scopolamine and benztropine (5 - 80 mg/kg, i.p.) were also ineffective against oxotremorine. The muscarinic antagonist -insensitive neurotoxicity of oxotremorine may involve nicotinic cholinergic receptors.

A similar atropine-insensitive neurotoxicity may have occurred in four squirrel monkeys, where oxotremorine (1.7 or 3 mg/kg, i.m.) produced death (24 hrs) despite treatment with sufficient atropine (2 mg/kg) to prevent the peripheral parasympathetic actions of oxotremorine and its effects on schedule-controlled behavior. In the absence of atropine, 0.3 mg/kg oxotremorine produced marked peripheral parasympathetic symptoms and disrupted ongoing behavioral performances, however, despite these profound actions, recovery was complete within several hours.

Taken as a whole, these findings suggest that many of the complications seen after exposure to nerve agents such as soman also occur after high doses of a relatively pure muscarinic agonist and, as with soman, such neurotoxicity is generally unresponsive to muscarinic receptor blockade. Since oxotremorine and related compounds have more circumscribed actions than those of conventional nerve agents, these compounds may provide crucial insights into the mechanisms responsible for nerve agent neurotoxicity and in the development of antidotal and prophylatic treatments.

INTRODUCTION

Oxotremorine is a highly selective ligand of the muscarinic cholinergic receptor in the central and peripheral nervous systems and is widely used as a tool in investigations of muscarinic functions. Oxotremorine produces signs of parasympathetic activation and produces central nervous system actions which are in accord with the idea that the predominant pharmacology of oxotremorine is based upon binding to muscarinic receptors (1, 2, 3, 4). This report documents that relatively high doses of oxotremorine produce neurotoxic actions which are unresponsive to muscarinic antagonists.

METHOD

Male Sprague-Dawley rats (Zivic-Miller) weighing between 250 and 350 g were observed for various signs of muscarinic activation after administration of oxotremorine sesquifumarate (Aldrich Chemical Co.). Animals were treated with either saline or atropine sulfate (Sigma Chemical Co.), i.p., 15 min prior to oxotremorine, i.p. Doses are expressed as the salts. Observations were made for the presence or absence of signs by two observers, one blind to the treatments.

RESULTS

Oxotremorine produced dose- and time-dependent increases in lacrimation, salivation, tremor, convulsions and death (Figs. 1 - 4). Onset of action was rapid and dose-effect functions were steep. Although relatively low doses of oxotremorine (2.5 mg/kg and below) produced lacrimation, salivation and tremor, no convulsions or death were observed (Fig. 1).

All observed effects of oxotremorine at 5 mg/kg and below were completely prevented by atropine pretreatment (Fig. 2). In contrast, higher doses of oxotremorine (7.5 - 20 mg/kg) produced convulsions that were not affected by atropine. Lacrimation and salivation, however, were absent in atropine-treated rats whereas tremor and death were still prevalent (Fig. 3). Although death only occurred in convulsing animals, lethal effects of oxotremorine were differentiated from its convulsive effects by atropine pretreatment.

Generalized clonic convulsions were characterized by relatively disorganized sequences of motor patterns. Myoclonic jerking of the upper body was common, and during some stages, the convulsions resembled those associated with partial seizure activity, with stereotyped head bobbing. Convulsive episodes were preceded and followed by maintenance of a flat, prone posture.

The inability of atropine to prevent oxotremorine-induced neurotoxicity was not due the use of insufficient concentrations of atropine, time parameters, or routes of administration. Doses of atropine from 5 to 160 mg/kg, i.p., or 40 mg/kg atropine, s.c., were also ineffective; administration of atropine at 15, 30, 45 or 60 min prior to oxotremorine or atropine administered during convulsive episodes was also inadequate to protect or reverse neurotoxic actions of oxotremorine. Pretreatment of rats with scopolamine or benztropine (5 - 80 mg/kg, i.p., 15 min prior) was also inadequate (data not shown).

Dose-dependent increases in lethality of oxotrmorine were observed. The LD-50 for oxotremorine was 8.3 mg/kg. The lethality function was shifted to the right in atropine-treated rats (Fig. 4). Death was preceded by cyanosis.

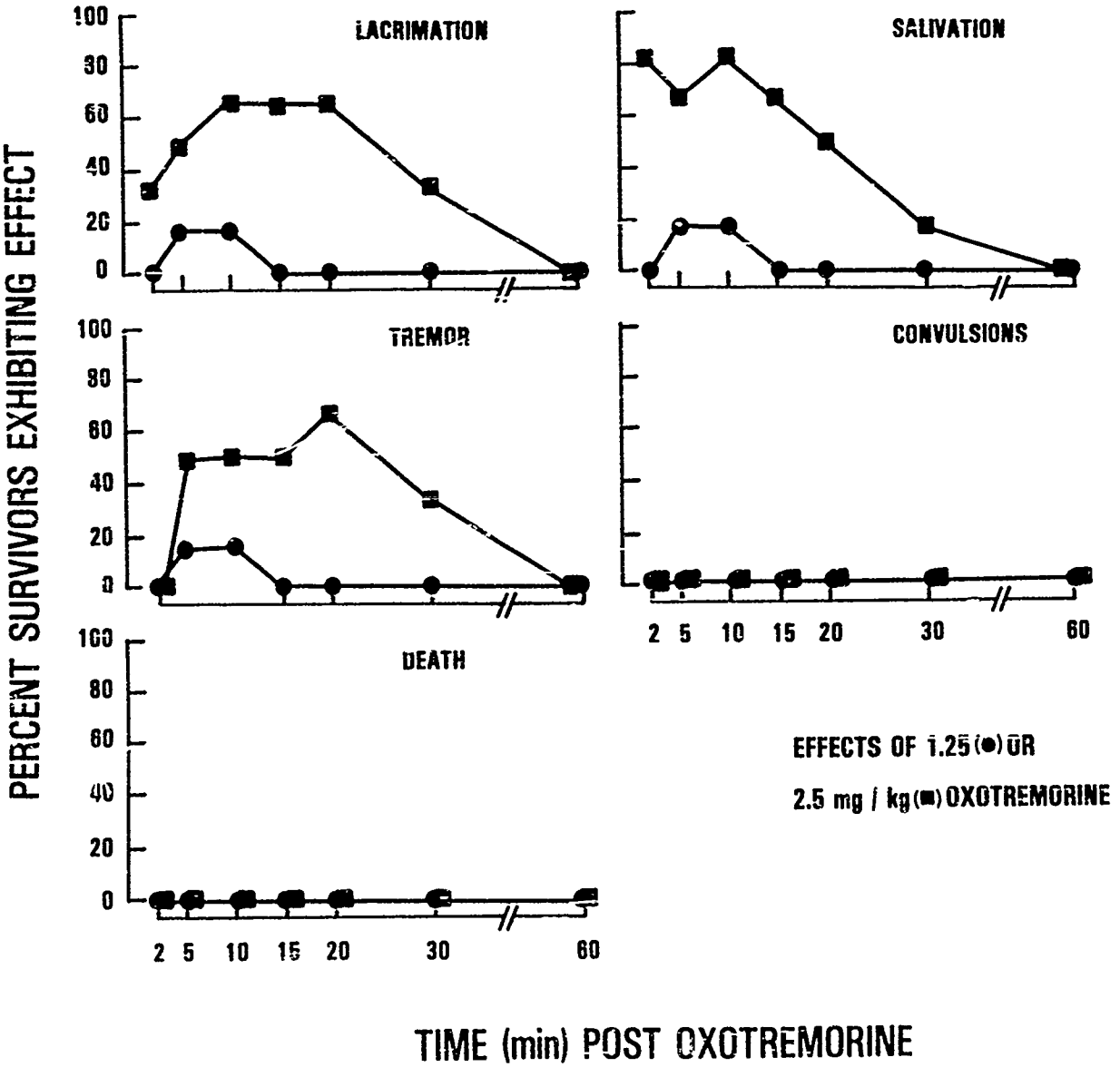

FIGURE 1. Oxotremorine produced dose- and time-dependent increases in signs of muscarinic activation. The relatively low doses shown in this figure (1.25 and 2.5 mg/kg) did not produce convulsions or death in any of the animals tested. Six animals were studied at each dose.

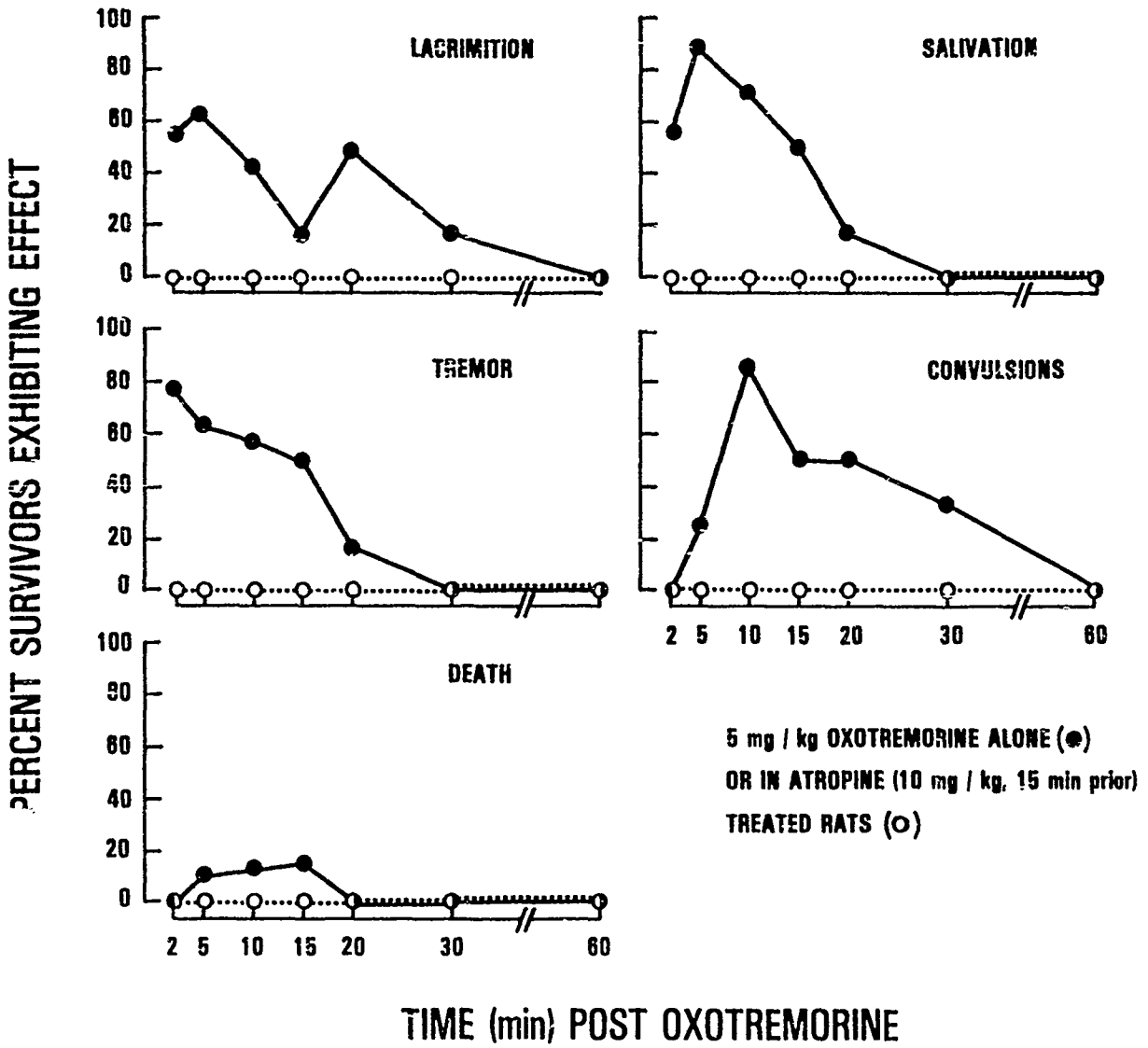

FIGURE 2. Effects of relatively low neurotoxic doses of oxotremorine (eg. 5 mg/kg) are completely prevented by atropine pretreatment. Nine animals were studied with oxotremorine alone and nine were observed after atropine (10 mg/kg) treatment. Atropine alone did not affect any of these measures.

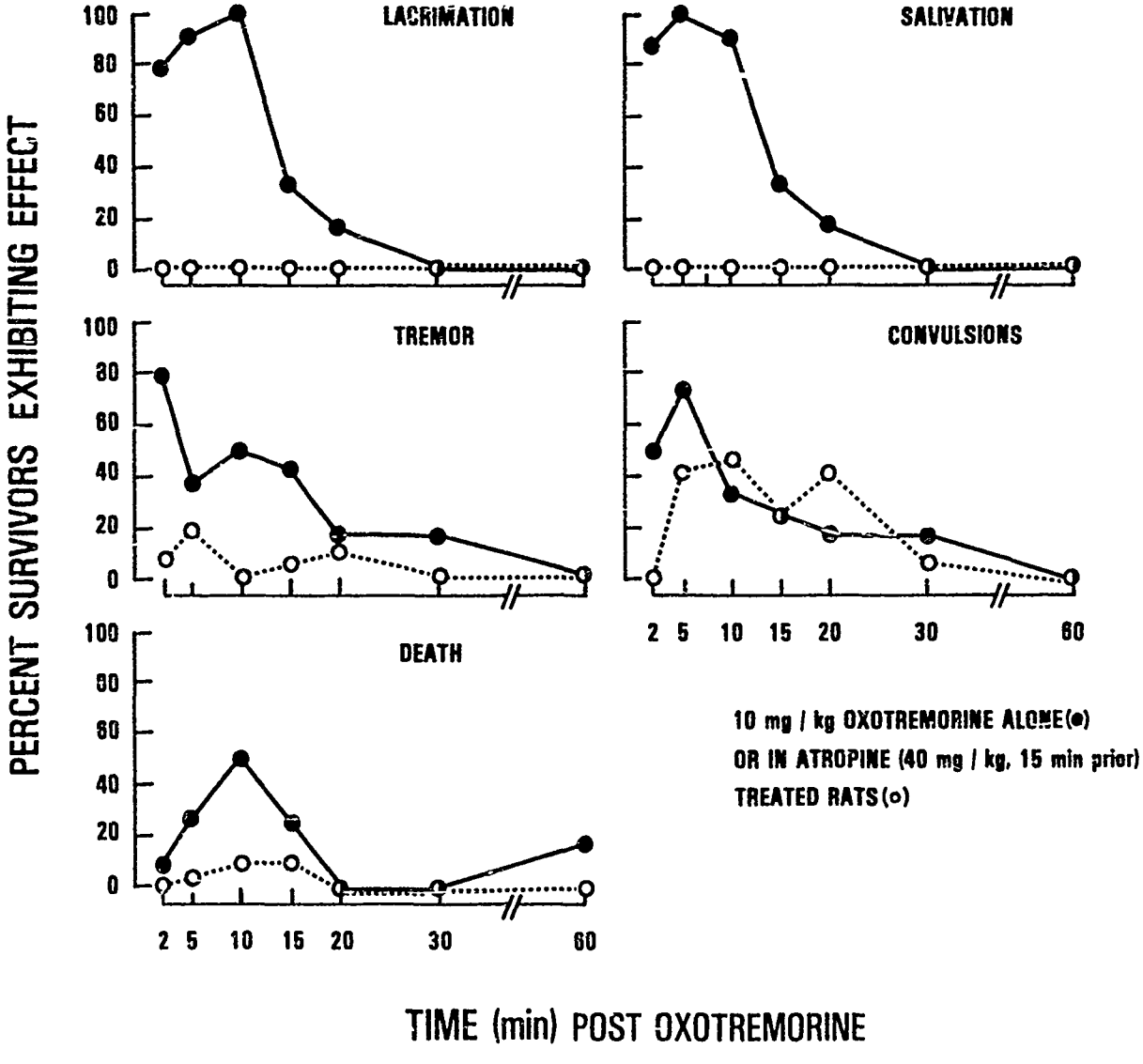

FIGURE 3. Doses of oxotremorine higher than 5 mg/kg produced convulsions that were unresponsive to atropine pretreatment; likewise, tremor and death could not be completely prevented with atropine. Lacrimation and salivation were totally absent in atropine-treated rats. Atropine alone was without affect on these measures. Note that the dose of atropine is 4 times higher than necessary to prevent all manifestations of oxotremorine-induced neurotoxicity at 5 mg/kg (Fig. 2). Twenty four rats were observed after oxotremorine alone and twenty two atropine-treated animals were studied.

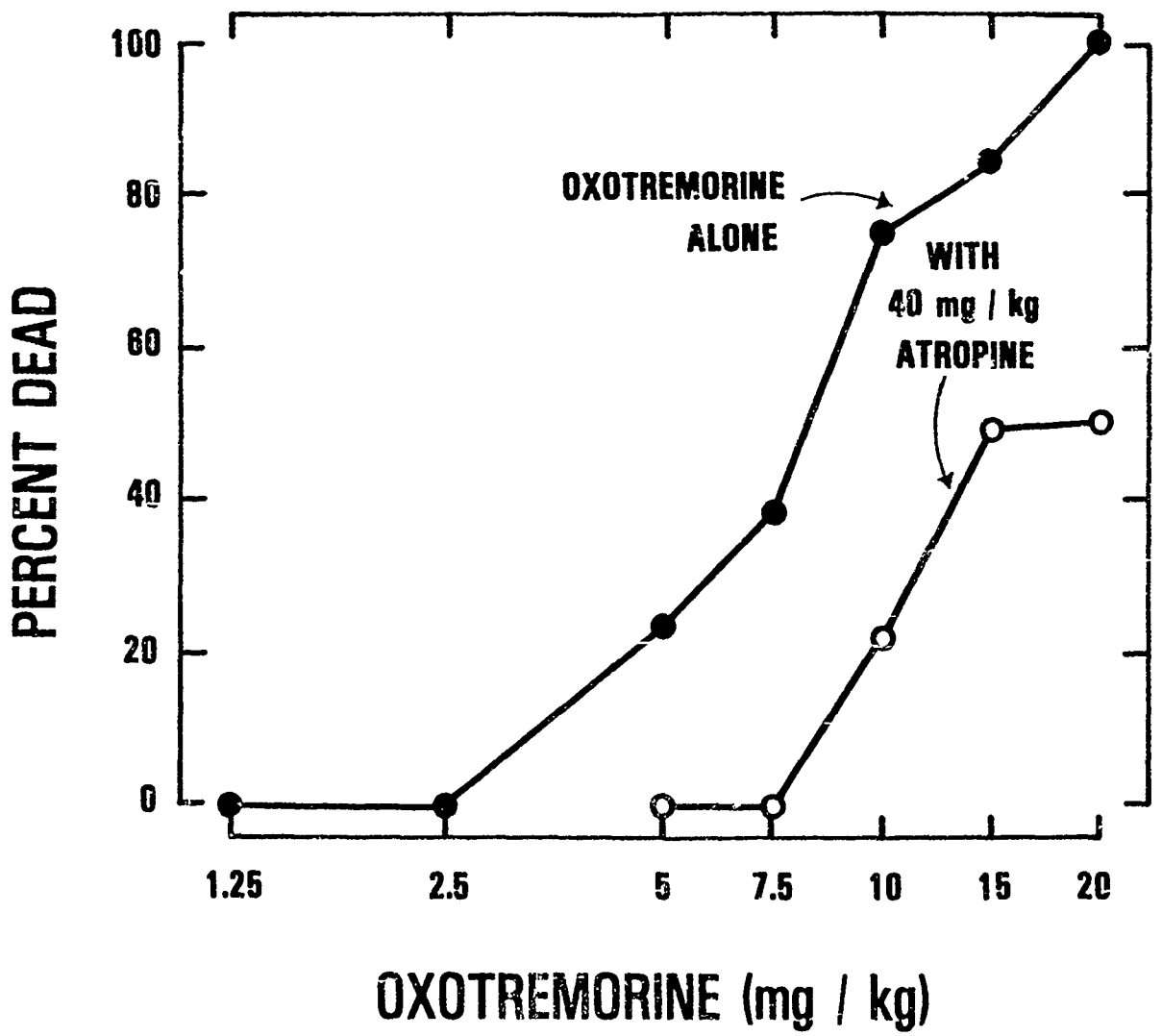

FIGURE 4. Atropine pretreatment altered the lethal effects of oxotremorine. Percent of animals killed was measured at 60 min.

CONCLUSION

Two types of dose-dependent oxotremorine neurotoxicity were observed in this study, one responsive to atropine and one insensitive to atropine. The neurotoxicity of relatively high doses of oxotremorine appears to be unrelated to actions of oxotremorine at muscarinic receptors since, in addition to atropine, scopolamine and benztropine were also ineffective against oxotremorine.

Atropine-insensitive neurotoxicity of oxotremorine may involve nicotinic cholinergic receptors. Possible involvement of oxotremorine at peripheral and central nervous system nicotinic sites has been proposed (cf. 5, 6).

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5. Ganguly, D.K. and S.K. Chaudhuri (1970). Eur. J. Pharmacol. 11: 84-89.
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ROLE OF ACETYLCHOLINESTERASE IN CHOLINERGIC SYNAPTIC TRANSMISSION

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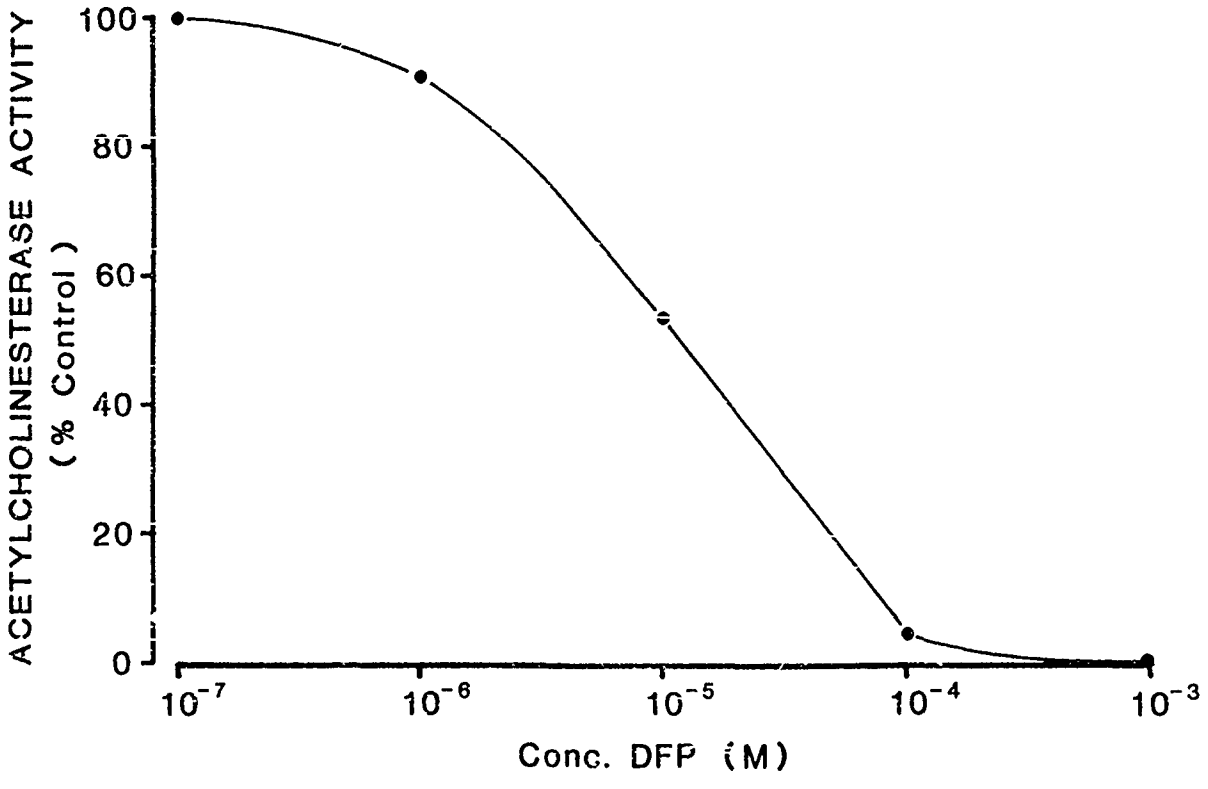
INTRODUCTION

A cell culture system was utilized to investigate the actions of acetylcholinesterase (AChE) inhibitors on cholinergic synaptic transmission. The presynaptic element consisted of the neuroblastoma x glioma hybrid cell NG108-15 which releases acetylcholine (ACh); the postsynaptic element was subserved by the clonal G8-1 myotube, which expresses nicotinic ACh receptors in high density but has low AChE activity. When grown in co-culture, NG108-15 neurites terminate on myotube receptor clusters and form functional cholinergic synapses. Transmitter release in the biclonal system is quantal in nature and Ca^{2+}-dependent. By morphological criteria, the NG108-15/G8-1 synapses resemble those appearing during the early stages of neuromuscular embryogenesis. The clonal syanpses are devoid of junctional folds, connective tissue and Schwann cell processes. The primary cleft shows a 20--50 nm gap with discontinuous synaptic basal lamina.

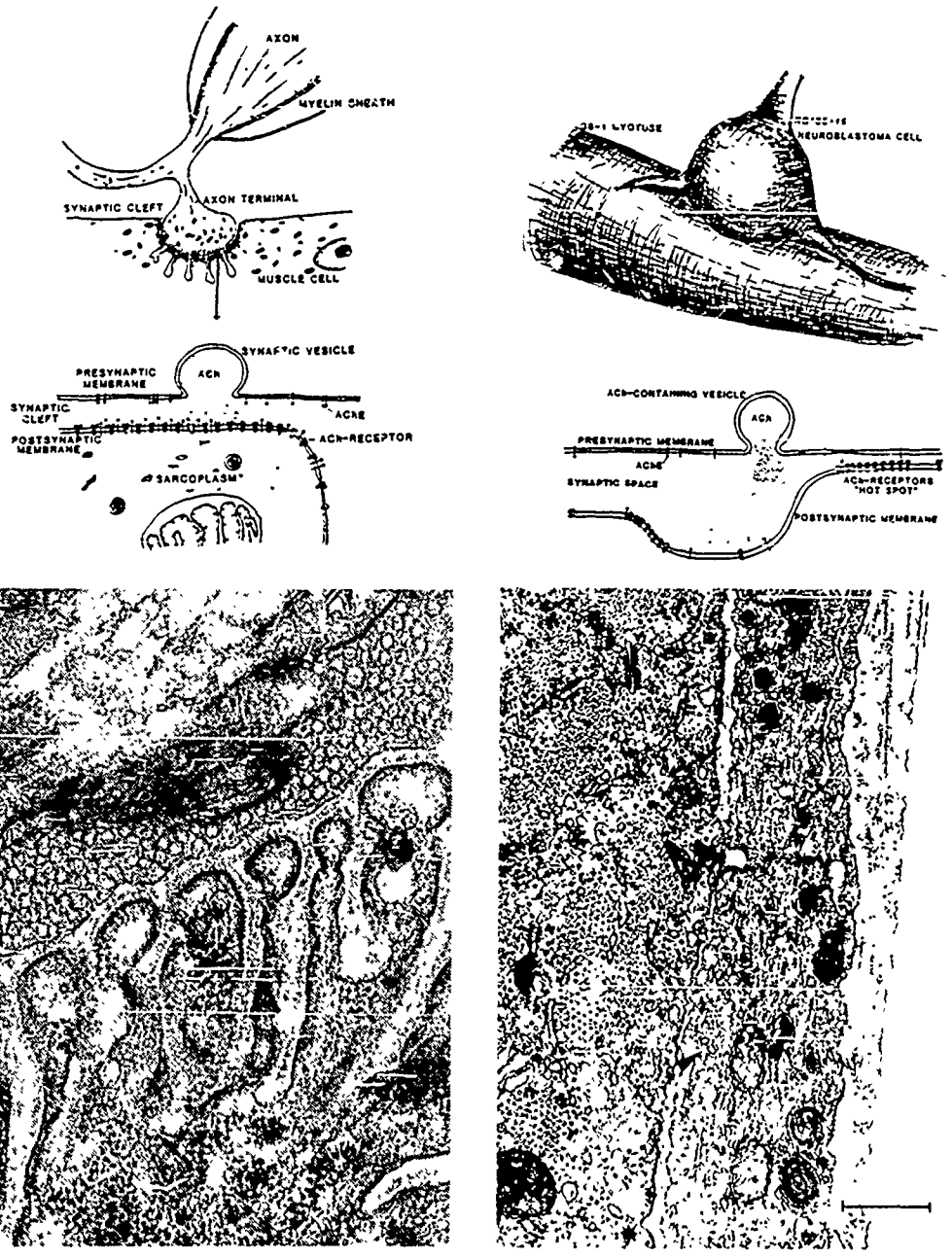
Our previous work has demonstrated some direct effects of the irreversible organophosphorous agent diisopropylfluorophosphate (DFP) on the ACh receptor-channel macromolecule. These consisted of a depression in the miniature synaptic potential (MSP) amplitude , a shortening in the open channel lifetimes and an enhancement in the desensitization rate. However, washout of unreacted DFP did not lead to the typical increases in the synaptic potential decay phase that accompany inhibition of AChE at the motor endplate. In this study we have used patch and whole-cell voltage-clamp techniques to determine why clonal synapses are relatively insensitive to AChE inhibition.

METHODS

Co-cultures were established by seeding 8-10 day old myotube dishes with 5×10^4 NG108-15 cells and maintained in DMEM containing 10% horse serum, 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine and 10^{-3} M dibutyryl cyclic-AMP. Patch-clamp or whole-cell voltage-clamp recordings were carried out after at least three days in co-culture. Single ACh-activated channels were obtained on outside-out patches at -80 mV and 37 ^{o}C.

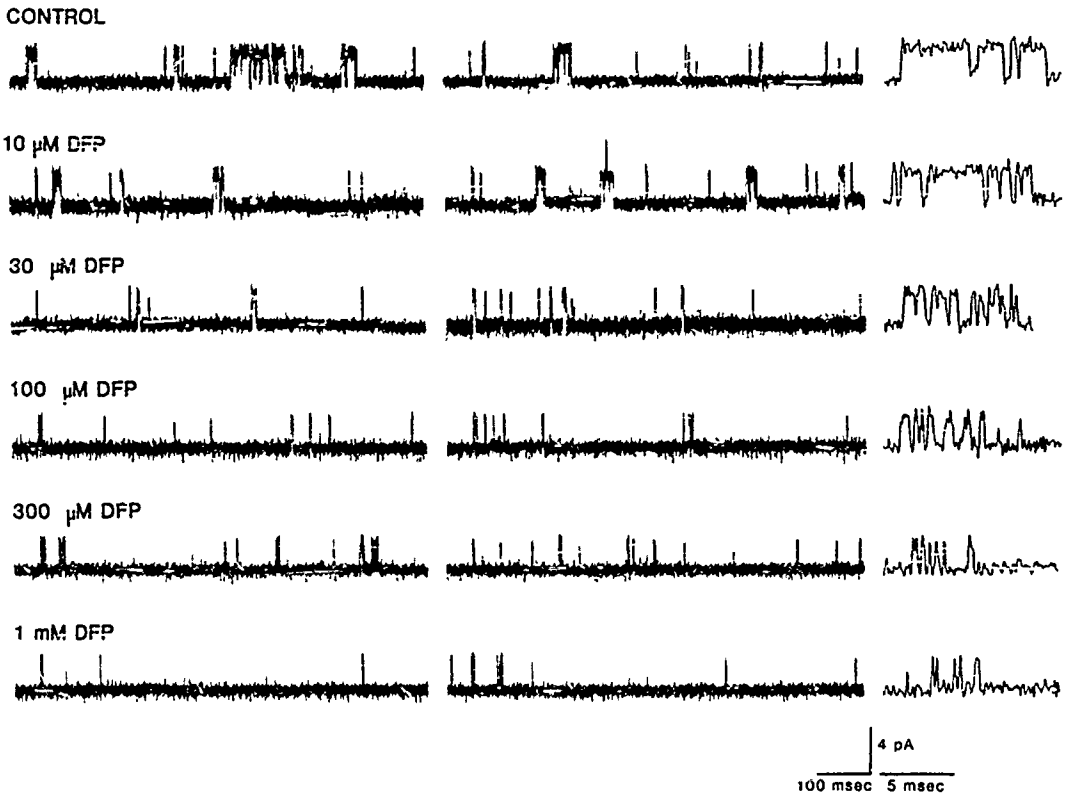


Dose-response curve for the inhibition of G8-1 acetylcholinesterase activity by diisopropylfluorophosphate (DFP). The 100% point corresponds to an activity of 0.83 mmole/min/mg protein. DFP was added to the supernatant fraction of Triton X-100 homogenates for 30 min prior to assay.

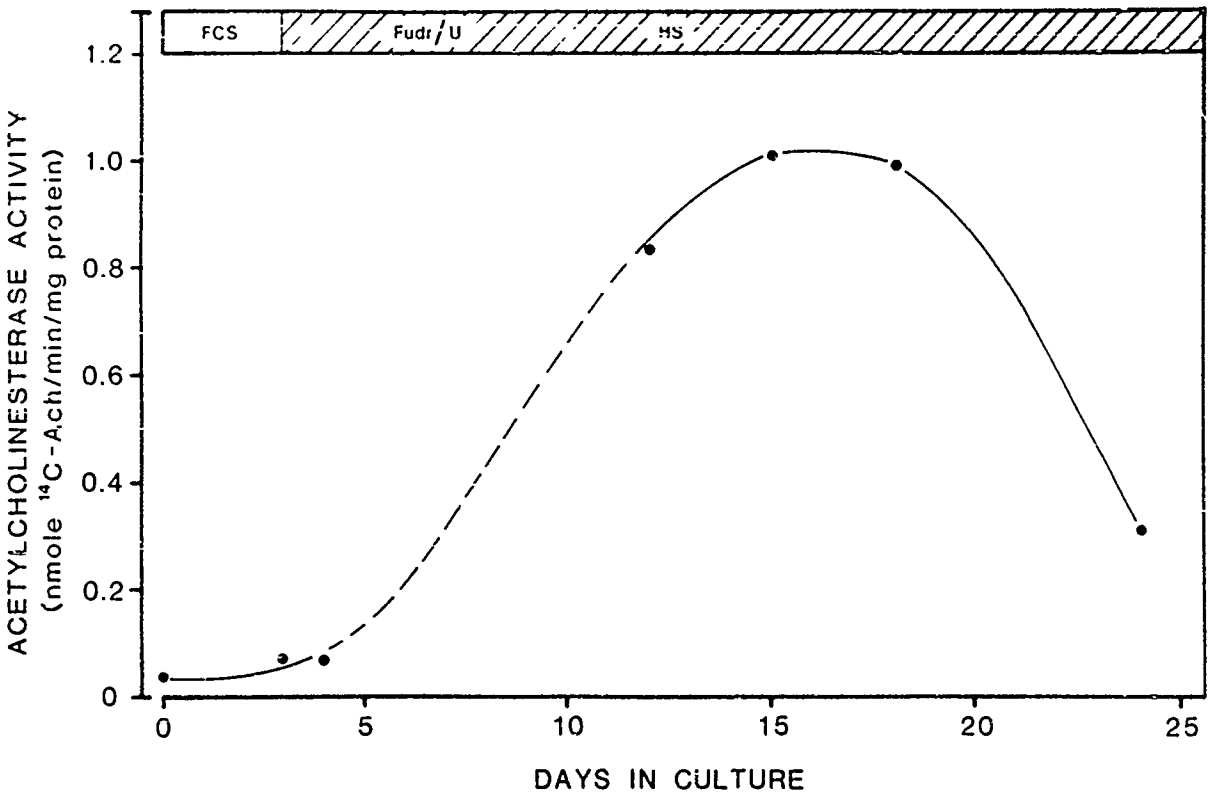


Morphological characteristics of mature neuromuscular synapse (left) and clonalsynapse (right). Upper panels are schematic diagrams showing the relationships between transmitter release sites and receptor clusters. Imprecise alignment between release sites and receptor hot spots is considered to underly the vaiability in the MSC kinetics. Lower panels are electron micrographs of a neuromuscular junction from rat diapgragm (60,000X, left) and an NG108-15/Myotube synapse (44,000X, left). For the latter, note paucity of clear synaptic vesicles, the presence of dense cored granules, the variable primary cleft, the absence of junctional folds and the discontinuities in the basal lamina.

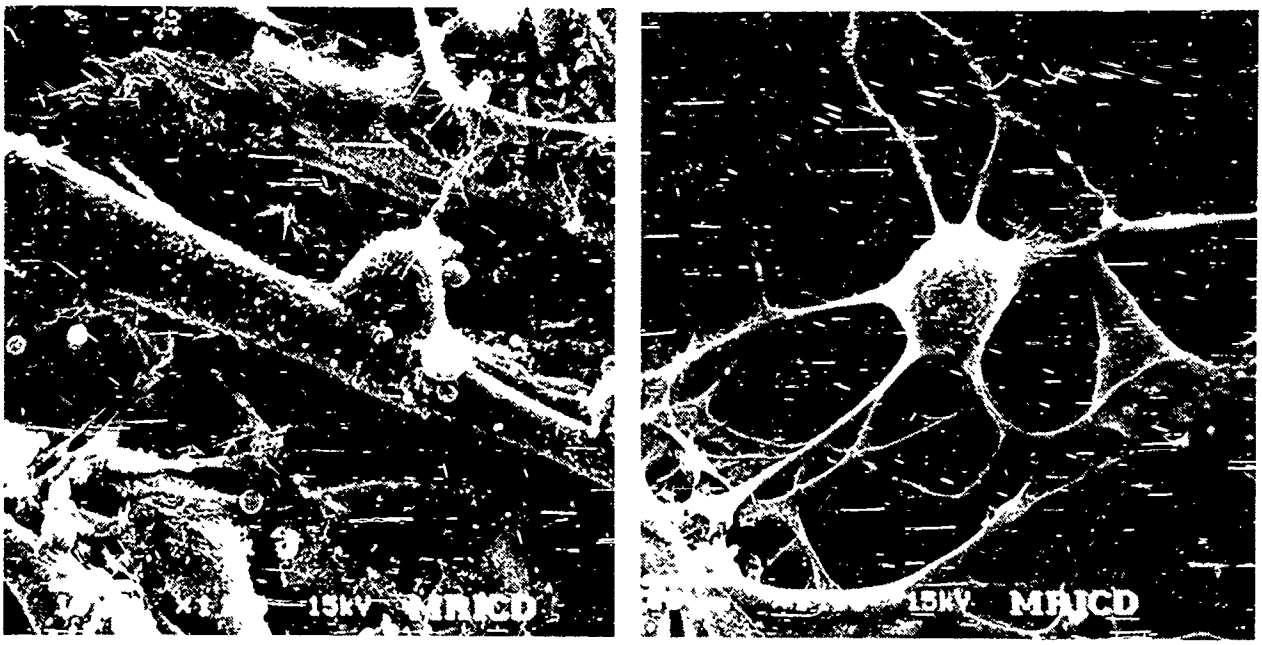
EFFECT OF DFP ON SINGLE MYOTUBE ACETYLCHOLINE CHANNEL CURRENTS



Single channel currents from an excised outside-out patch held at -80 mV in the control solution and in the presence of increasing concentrations of DFP: Acetylcholine (0.5 uM) was present throughout. Upward deflection denotes inward current.



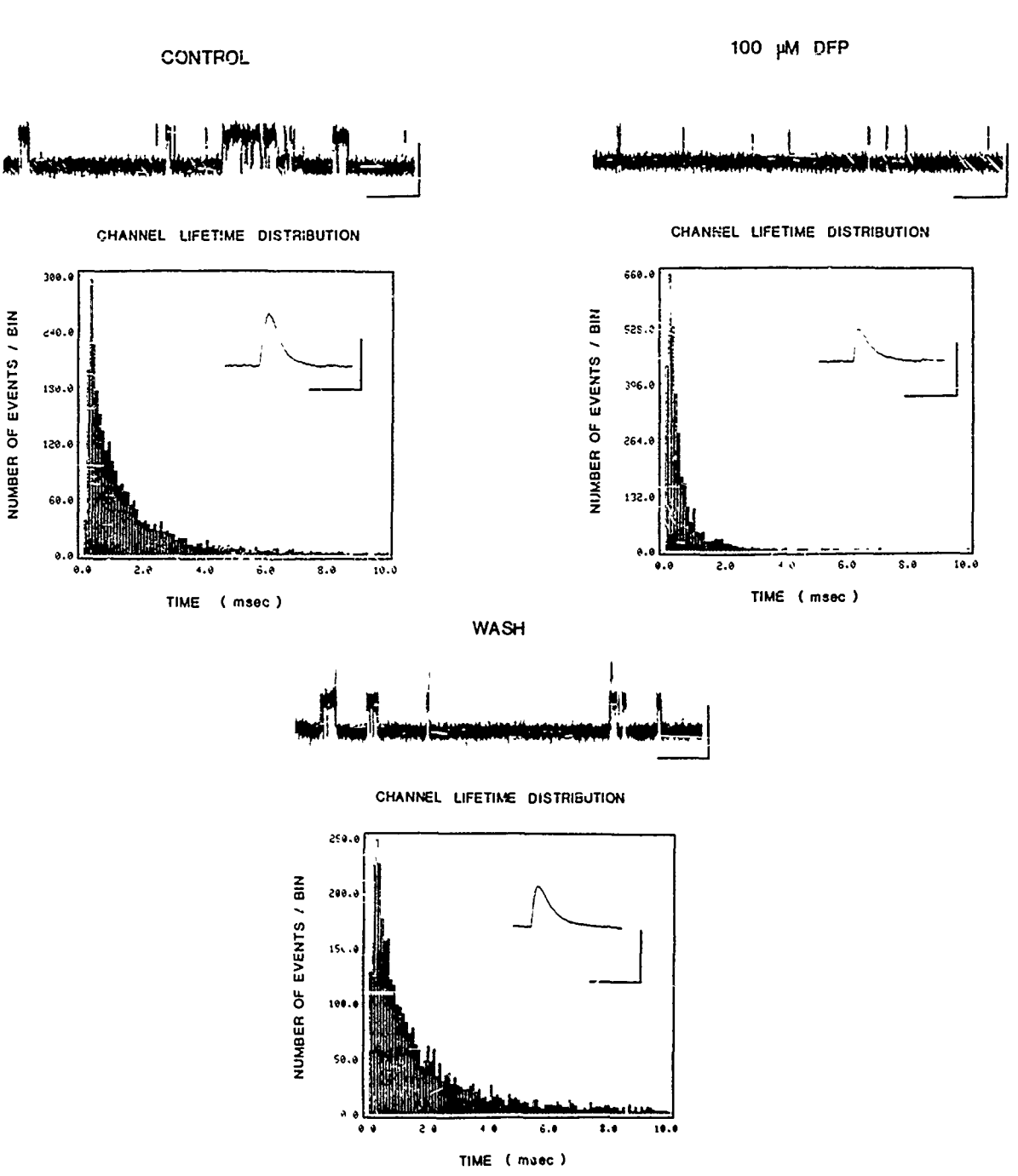
Development of actylcholinesterase activity in G8-1 cultures. Cells were homogenized in 1% Triton X-100 and centrifuged at 12,000x g for 15 min. Acetylcholinesterase activity was assayed in the supernatant using 1 mM ^{14}C-Acetylcholine in 0.1 M potassium phosphate buffer (pH 7.4) at 37 ^{o}C. Protein content was determined by the Coomassie blue dye-binding assay using albumin as a standard. The cell were maintained in DMEM with fetal calf serum (FCS) for 4 days, then switched to horse serum (HS) for the remaining period. 5'-Fluorodeoxyuridine (10^{-4} M) and Uridine (10^{-4} M) (Fudr/U) were added for the indicated time to inhibit further myoblast proliferation.



SCANNING ELECTRON MICROGRAPHS SHOWING TWO COMMON MODES OF SYNAPTIC CONTACT BETWEEN NG108-15 AND G8-1 MYOTUBES.

LEFT: THE NEURONAL SOMA APPEARS TO MAKE EXTENSIVE CONTACT WITH THE MYOTUBE MEMBRANE.

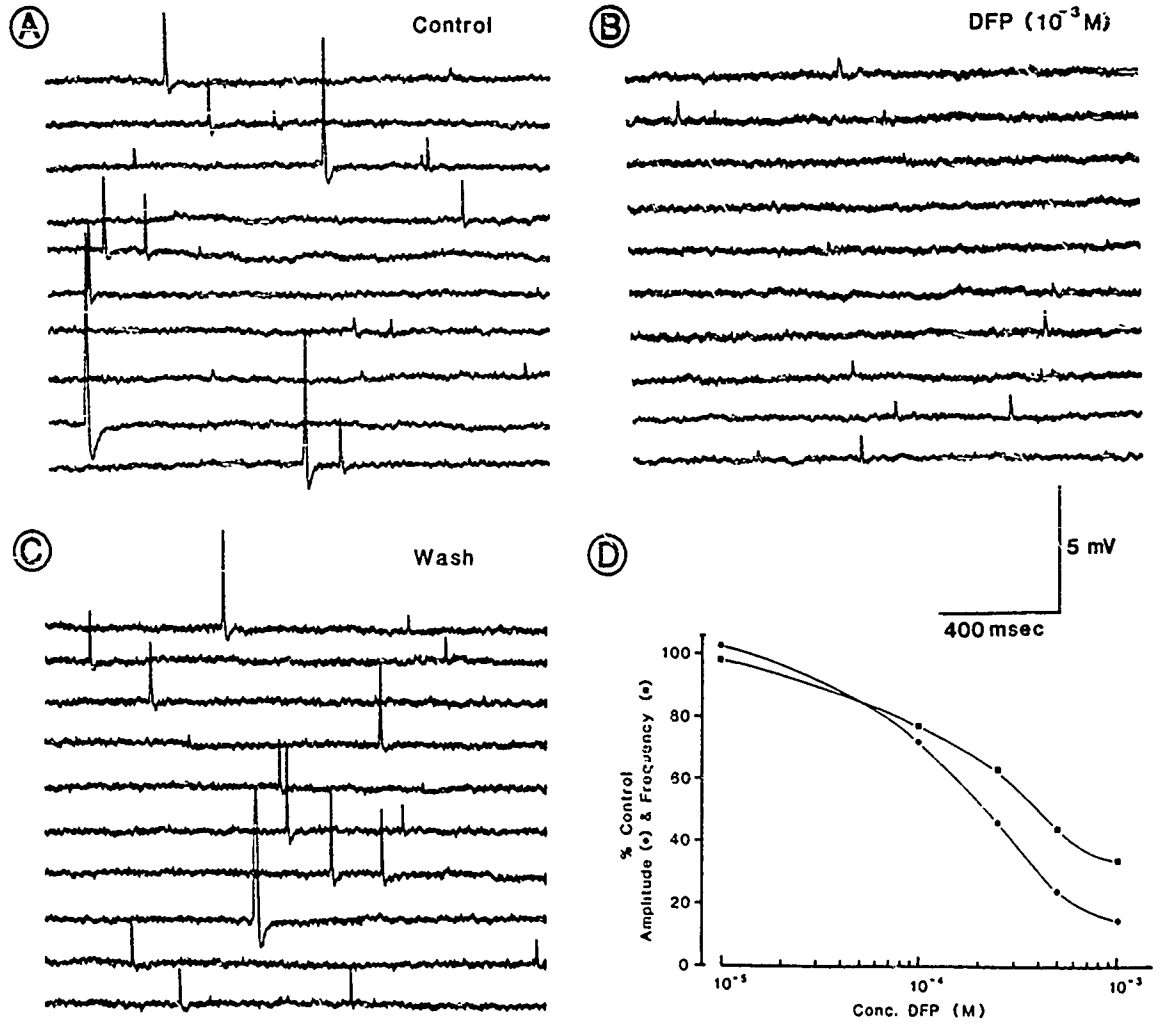
RIGHT: AN NG108-15 NEURITE IN APPARENT SYNAPTIC CONTACT WITH A MYOTUBE. THE REGION OF NERVE-MUSCLE CONTACTS ARE ASSOCIATED WITH HIGH ACETYLCHOLINE RECEPTOR DENSITIES.



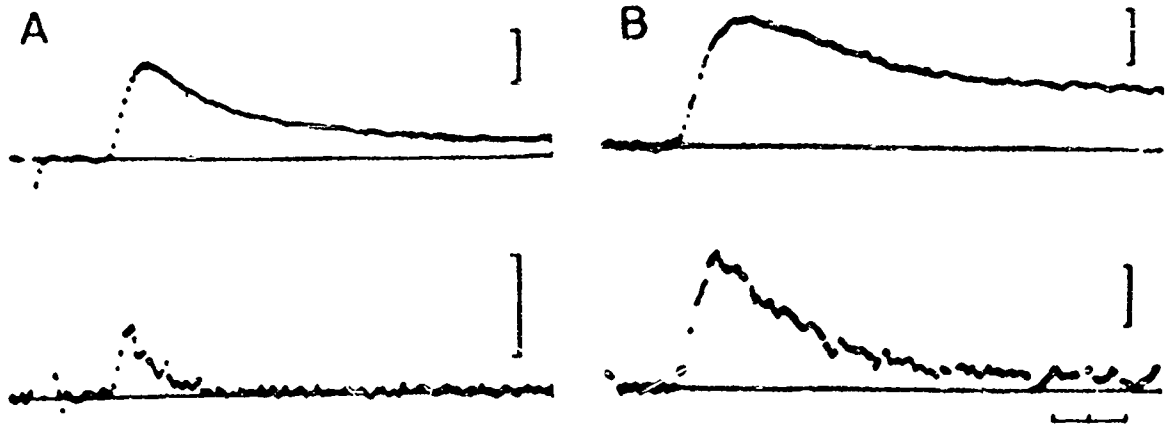
EFFECTS OF DIISOPROPYLFLUOROPHOSPHATE (DFP) ON SYNAPTIC AND SINGLE CHANNEL CONDUCTANCES. THE CONDITIONS INDICATED ARE CONTROL, 45 MIN AFTER ADDITION OF 10^{-4} M DFP AND 45 MIN AFTER WASHOUT OF UNREACTED ORGANOPHOSPHATE. THE UPPER TRACES IN EACH PANEL REPRESENT SINGLE CHANNEL RECORDINGS FROM OUTSIDE PATCHES CLAMPED TO -80 mV IN THE PRESENCE OF 200 nM ACH. THE PLOTS SHOW THE EFFECTS OF DFP ON OPEN TIME DISTRIBUTION OF ACH-INDUCED CHANNELS. THE INSETS REPRESENT QUANTAL MINIATURE EXCITATORY SYNAPTIC CURRENTS (MSC'S) OBTAINED BY WHOLE-CELL VOLTAGE-CLAMP TECHNIQUES UNDER IDENTICAL CONDITIONS. INWARD CURRENT IS DENOTED BY AN UPWARD DEFLECTION.

CALIBRATION. (A) PATCH RECORDS: CURRENT, 6 pA; TIME, 15 msec.
(B) MSC'S: CURRENT, 200 pA; TIME, 6 msec.
TEMPERATURE: 37^{0} C.

		MEAN CHANNEL LIFETIMES	MSC HALF DECAY TIMES (msec)	DECAY TIME CONSTANT
CONTROL:		1.82	1.94	2.72
DFP:	100 μM	1.01	2.24	3.14
	300 μM	0.60	1.84	2.58
WASH:		1.78	2.10	2.94



Effect of DFP on spontaneous miniature synaptic potential (MSPs) in NG108-15/G8-1 co-cultures. The data were obtained from a 14 day old myotube under the indicated conditions. The traces in A and B are continuous. In C, the first 5 traces were taken 10 min after the onset of wash; the latter 5 were taken 40 min later. After-polarization of MSPs were observed in most cells and reversed at approximately -80 mV. D: Dose-response curve showing the DFP-induced inhibition in the MSP amplitude and frequency.



MODIFICATION OF THE ENDPLATE POTENTIAL (EPP, UPPER TRACE) AND ENDPLATE CURRENT (EPC, LOWER TRACE) BY PHYSOSTIGMINE IN A CURARIZED FROG SARTORIUS PREPARATION.

A. RECORDS OBTAINED PRIOR TO DRUG ADDITION.

B. RECORDS FROM THE SAME SYNAPSE AFTER EQUILIBRATION WITH 2 mM PHYSOSTIGMINE. THE INCREASE IN AMPLITUDE AND PROLONGATION OF THE DECAY HAS BEEN OBSERVED WITH ALL SKELETAL MUSCLE PREPARATIONS AND ALL ACETYLCHOLINESTERASE INHIBITIONS.

CALIBRATION: VOLTAGE, 2mV; CURRENT, 10^{-6} A; time, 2 msec.

SUMMARY AND CONCLUSIONS

1. The decay phase of the quantal miniature synaptic current (MSC) was found to be 1.5 to 5 times longer than the single channel open lifetime. This indicates that AChE is not sufficiently concentrated or correctly localized in the clonal synapse.

2. Blockade of AChE produced little additional prolongation of the MSC decay, suggesting that AChE does not contribute appreciably to the removal of transmitter from the synaptic cleft.

3. As in adult skeletal muscle, diffusional dilution of transmitter is not sufficiently rapid to maintain the synaptic current duration within the limits imposed by the channel gating time. This implies the existence of functional diffusion barriers.

4. Considerations of the morphological and physiological characteristics of the NG108-15 synapse suggests that the main barrier is the confinement of agonist molecules by the primary synaptic cleft in a region of high receptor density.

5. Receptor binding will slow the diffusion of ACh from the synaptic cleft by the factor:

$$\frac{(1 + R_T)}{(K_{-1})/(K_1)}$$

Where R_T represents the total number of receptors and $(K_{-1})/(K_1)$ is the agonist equilibrium dissociation constant. The MSC decay time constant, TAU_{MSC}, will then be determined by the above and the ACh diffusion rate (K_d) as

$$TAU_{MSC} = \frac{1 + \frac{[R_T]}{(K_{-1})/(K_1)} + \frac{K_d}{K_{-1}}}{K_d}$$

EFFECTS OF SOMAN ON THE REGIONAL DISTRIBUTION OF GLUTAMATE AND ACETYLCHOLINESTERASE IN RAT BRAIN

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ABSTRACT

EXPOSURE OF MICE TO CONVULSIVE DOSES OF SOMAN ELEVATES CEREBRAL CORTICAL GLUTAMATE IN THE PRESENCE OF ELEVATED HIGH ENERGY PHOSPHATE CONTENT (VIANA AND KAUFFMAN, NEUROSCI. ABS. 9: 210.19, 1983). SINCE GLUTAMATE IS A PUTATIVE EXCITATORY NEUROTRANSMITTER, ELEVATIONS IN THIS AMINO ACID COULD CONTRIBUTE TO THE NEUROTOXIC EFFECTS OF SOMAN. THUS, WE EXAMINED THE ACTIONS OF THIS ORGANOPHOSPHATE ON THE REGIONAL DISTRIBUTION OF GLUTAMATE IN RAT BRAIN. AREAS SELECTED FOR STUDY INCLUDED THE CEREBRAL CORTEX, CAUDATE NUCLEUS, NUCLEUS LOCUS COERULEUS, GLOBUS PALLIDUS, NUCLEUS AMBIGUOUS, NUCLEUS ACCUMBENS, AND NUCLEUS TRACTUS SOLITARIUS. A RECENT RADIOAUTOGRAPHIC STUDY OF THE DISTRIBUTION OF SOMAN LABELLED WITH C^{14} IN THE METHYLPHOSPHORYL PORTION OF THE MOLECULE, INDICATED PREFERENTIAL ACCUMULATION OF THE DRUG IN THE CAUDATE AND NUCLEUS ACCUMBENS (TRAUB ET AL., NEUROSCI. ABS. 10: 162.1, 1984).

ADULT MALE RATS WERE TREATED WITH EITHER 20 UG SOMAN/KG (0.75 OF THE LD50) AND KILLED BY DECAPITATION 30 MIN LATER OR 100 UG/KG AND KILLED 5 MIN AFTER THE ONSET OF TREMORS. BRAINS WERE IMMEDIATELY REMOVED, CHILLED ON ICE, MOUNTED ON CRYOSTAT CHUCKS AND FROZEN IN A DRY ICE - ACETONE MIXTURE. SECTIONS (20 UM) WERE PREPARED FROM THE MOUNTED TISSUES USING A CRYOSTAT MICROTOME MAINTAINED AT -20°. FROZEN CRYOSTAT SECTIONS WERE COLLECTED IN SPECIAL HOLDERS AND LYOPHILIZED AT -40° TO MAINTAIN HISTOLOGICAL STRUCTURE. ALTERNATE SERIAL SECTIONS WERE TAKEN FOR ACETYLCHOLINESTERASE, NISSL, AND MONOAMINE OXIDASE STAINING. THE LATTER WAS USED TO LOCALIZED THE NUCLEUS LOCUS COERULEUS. SAMPLES WEIGHING BETWEEN 0.2 AND 2.5 UG DRY WEIGHT WERE MICRODISSECTED FROM LYOPHILIZED SECTIONS USING QUANTITATIVE HISTOCHEMICAL PROCEDURES (LOWRY, J. HISTOCHEM. CYTOCHEM. 1: 420, 1953) MICROBIOCHEMICAL MEASUREMENTS OF GLUTAMATE (AUSTIN ET AL., J. BIOL. CHEM. 126: 351, 1972) AND ACETYLCHOLINESTERASE (JOHNSON AND RUSSELL, ANAL. BIOCHEM. 64: 229, 1975) WERE MADE ON MICRODISSECTED SAMPLES FROM THE VARIOUS BRAIN REGIONS.

ADMINISTRATION OF THE LOW DOSE OF SOMAN (20 UG/KG) RESULTED IN A SIGNIFICANT DECREASE IN ACETYLCHOLINESTERASE IN THE NUCLEUS LOCUS COERULEUS (58%) AND ELEVATIONS IN THE ACTIVITY OF THIS ENZYME IN CHOLINERGIC NUCLEI SUCH AS THE ISLANDS OF CALLEJA (100%) AND THE NUCLEUS OF THE HYPOGLOSSAL NERVE (230%). ANIMALS TREATED WITH THE HIGHER DOSE OF SOMAN (100 UG/KG) HAD ESSENTIALLY NO ACETYLCHOLINESTERASE IN MOST BRAIN AREAS; HOWEVER, THE ENZYME REMAINED ACTIVE IN CERTAIN NUCLEI. AMONG THESE WERE THE ISLANDS OF CALLEJA, THE LATERAL CAUDATE-PUTAMEN, THE NUCLEUS OF THE HYPOGLOSSAL NERVE, THE NUCLEUS LOCUS COERULEUS, AND THE RETICULAR FORMATION. GLUTAMATE CONTENT WAS ELEVATED IN THE CEREBRAL CORTEX, BOTH AFTER THE ADMINISTRATION OF THE LOW DOSE OF SOMAN (65%) AND OF THE HIGH DOSE (49%) AND IN THE CAUDATE-PUTAMEN (45% AFTER A LOW DOSE). THESE RESULTS INDICATE THAT SOMAN MAY ELEVATE SELECTIVELY GLUTAMATE IN THE CEREBRAL CORTEX. THE EFFECTS OF SOMAN ON BRAIN ACETYLCHOLINESTERASE ARE BY NO MEANS UNIFORM AND DO NOT NECESSARILY CORRELATE WITH BINDING OF RADIOACTIVE SOMAN.

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INTRODUCTION

ORGANOPHOSPHATES CAUSE CONVULSIONS AND CARDIOVASCULAR FAILURE, BUT THEIR MAIN ACUTE TOXICITY IS BELIEVED TO BE ASSOCIATED WITH HYPOXIA. THIS EFFECT IS RELATED TO INHIBITION OF AChE, AND CONSEQUENTLY ELEVATION OF ACETYLCHOLINE AT CENTRAL AND PERIPHERAL SYNAPSES, E.G., PHRENIC NERVE - DIAPHRAGM NEUROMUSCULAR JUNCTION. NEVERTHELESS, THERE ARE INDICATIONS THAT CENTRAL RESPIRATORY NEURONS MAY ALSO BE AFFECTED THROUGH AN ACTION OF THE AGENT ON NEUROTRANSMITTERS DIFFERENT FROM ACh.

THE BASIC RHYTHMICITY OF RESPIRATION IS CONTROLLED BY MEDULLARY RESPIRATORY CENTERS, SUCH AS NUCLEUS AMBIGUOUS, N. RETROAMBIGUOUS AND N. TRACTUS SOLITARIUS. THE LATTER IS SUGGESTED TO BE A RESPIRATORY PACESETTER SINCE IT SEND PROJECTIONS BOTH TO BRAIN STEM NEURONS AND TO DESCENDING STRUCTURES. GLUTAMATE IS POSTULATED TO BE A MAJOR EXCITATORY NEUROTRANSMITTER IN THE N. TRACTUS SOLITARIUS. GLUTAMATE EXHIBITS HIGH AFFINITY SYNAPTOSOMAL UPTAKE IN THE NUCLEUS AMBIGUOUS, THE MEDIAL SEPTUM AND THE HIPPOCAMPUS (PERRONE, M.H., BRAIN RES. 230: 283-293, 1981).

BASED ON THESE FACTS, AS WELL AS ON OBSERVED INCREASES IN GLUTAMATE CONTENT OF THE CEREBRAL CORTEX OF MICE GIVEN A LETHAL DOSE OF SOMAN, NEURONAL TOXICITY COULD BE EXPLAINED VIA ELEVATION IN THIS AMINO ACID. ACCORDINGLY, WE EXPLORED THIS POSSIBILITY AND WE EVALUATED THREE DIFFERENT TECHNIQUES TO MEASURE GLUTAMATE IN MICRODISSECTED SAMPLES OF THE RAT CENTRAL NERVOUS SYSTEM AND MEASURED THIS AMINO ACID IN A WIDE VARIETY OF STRUCTURES. ALTERATIONS IN GLUTAMATE WERE COMPARED WITH THE ACTION OF THE ORGANOPHOSPHATE ON THE ACTIVITY OF ACETYLCHOLINESTERASE.

METHODS

ADULT MALE RATS WERE INJECTED WITH SOMAN INTRAMUSCULARLY: 20 UG/KG AND DECAPITATED 30 MINUTES OR 48 HOURS LATER, OR 100 UG/KG AND DECAPITATED 5 MINUTES AFTER THE ONSET OF CONVULSIONS. BRAINS WERE IMMEDIATELY REMOVED, MOUNTED ON CRYOSTAT CHUCKS AND FROZEN IN A DRY ICE - ACETONE MIXTURE. 20 UM CRYOSTAT SECTIONS (CUT AT -20°C) WERE COLLECTED IN SPECIAL HOLDERS AND WERE LYOPHILIZED AT -40°C. ALTERNATE SECTIONS WERE TAKEN FOR ACETYLCHOLINESTERASE, NISSL AND MONOAMINE OXIDASE STAINING (EMPLOYED TO LOCALIZED THE NUCLEUS LOCUS COERULEUS). MICRODISSECTED SECTIONS FROM THE LYOPHILIZED TISSUE WEIGHING BETWEEN 0.2 AND 2.5 UG DRY WEIGHT WERE USED FOR THE MICROBIOCHEMICAL MEASUREMENTS OF GLUTAMATE AND ACETYLCHOLINESTERASE.

THREE METHODS FOR DETERMINING THE GLUTAMATE CONTENT WERE USED: A DIRECT ASSAY (LOWRY ET AL., J. BIOL. CHEM. 239: 18-30, 1964), AN ASSAY INVOLVING INDUCED FLUORESCENCE (LOWRY AND PASSONNEAU, A FLEXIBLE SYSTEM OF ENZYMATIC ANALYSIS, ACADEMIC PRESS, NEW YORK, 1972), AND A CYCLING ASSAY (AUSTIN ET AL., J. BIOL. CHEM. 126: 351, 1972).

ACETYLCHOLINESTERASE WAS DETERMINED USING TWO MICROASSAYS - A RADIOMETRIC ASSAY (JOHNSON AND RUSSELL, ANAL. BIOCHEM. 64: 229-238, 1975) AND A COLORIMETRIC ASSAY (ELLMAN ET AL., BIOCHEM. PHARMACOL. 7: 88-95, 1961).

METHODS USED TO DETERMINE GLUTAMATE

1. Direct Fluorometric Method

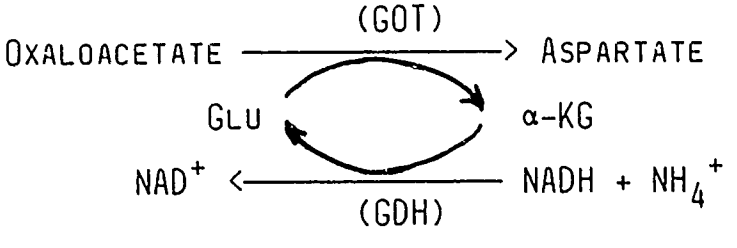
$$\text{Glu} + \text{NAD}^+ \xrightarrow[\text{GDH}]{} \alpha\text{-KG} + \text{NH}_4^+ + \text{NADH} + \text{H}^+$$

Microdissected samples and standards were dissolved in 0.1 N HCl and were incubated for 30 minutes in a reagent containing 125 mM Tris-acetate buffer (pH 8), 100 µM ADP, 300 µM NAD^+, 200 µg/ml BSA and 100 µg/ml glutamate dehydrogenase (GDH). The NADH formed was determined fluorometrically in 0.05 M carbonate, pH 10.3, using a Farrand filter fluorometer (340 --> 420 nm).

2. Alkali Enhanced Fluorescence Method

At the end of the incubation period, the reaction tubes were heated at 60°C for 15 min in a phosphate buffer at pH 12, destroying NAD^+. Then NADH was oxidized with 0.03% H_2O_2 in 6.5 N NaOH and the fluorescent product was developed by heating the solution at 60°C for 15 min in subdued lighting.

3. Cycling Microassay



SAMPLES AND STANDARDS WERE DISSOLVED IN 0.05 N NaOH AND WERE HEATED TO 90°C FOR 3-4 MIN TO DESTROY MALATE DEHYDROGENASE AND LACTATE DEHYDROGENASE. INCUBATION WAS PERFORMED FOR 1 HR IN 50 µL REAGENT CONTAINING 50 mM IMIDAZOLE BUFFER (pH 6.7), 10 mM NH_4 ACETATE, 0.02% BOVINE SERUM ALBUMIN. 0.2 mM ADP, 0.4 mM NADH, 0.4 mM OXALOACETATE, 5 µG/ML GLUTAMATE OXALOACETATE TRANSAMINASE AND 5 µG/ML GLUTAMATE DEHYDROGENASE. AT THE END OF THAT PERIOD, THE REMAINING NADH WAS DESTROYED WITH 0.5 N HCL AND FLUORESCENCE WAS DEVELOPED FROM NAD^+ BY HEATING THE REACTION MIXTURE AT 60°C FOR 10 MIN IN 6.5 N NaOH.

TABLE 1

CHARACTERISTICS OF THREE METHODS EMPLOYED IN THE EVALUATION OF THE GLUTAMATE CONTENT OF BRAIN TISSUE

TYPE OF ASSAY	LOWEST LEVEL OF GLUTAMATE DETECTED	RANGE OF LINEARITY	FLUORESCENCE (340 -> 420 NM)		SIGNAL BLANK
			BLANK	STANDARD (GLUTAMATE)	
DIRECT FLUORESCENCE	60 PMOLS	60-300 PMOLS	25.4 ± 0.3 (4)	27 -> 60 PMOLS 41 -> 300 PMOLS	1.06
ALKALI ENHANCED	10 PMOLS	10-130 PMOLS	67.3 ± 5.9 (6)	71 -> (10 PMOLS) 87 -> (130 PMOLS)	1.05
CYCLING ASSAY	5 PMOLS	5- 30 PMOLS	42.6 ± 2.4 (3)	50 (5 PMOLS) 64 (180 PMOLS)	1.17

EXAMPLES OF TYPICAL VALUES FOR EACH KIND OF ASSAY ARE BEING COMPARED. BLANKS ARE AVERAGES OF THE NUMBER OF REPLICATES MARKED IN PARENTHESES ± SD. THE FLUORESCENCE OF THE STANDARD IS SHOWN FOR BOTH THE LOWEST AND THE HIGHEST AMOUNT TESTED IN EACH PARTICULAR TYPE OF ASSAY. THE SIGNAL TO BLANK RATIO IS INDICATED FOR THE LOWEST AMOUNT OF GLUTAMATE DETECTED.

TABLE 2

GLUTAMATE CONTENT IN SPECIFIC BRAIN AREAS OF NORMAL AND SOMAN-TREATED RATS.
COMPARISON OF DIFFERENT ANALYTICAL METHODS

	CONTROL		20 UG/KG SOMAN		100 UG/KG SOMAN	
	GLUTAMATE	ASSAY TYPE	GLUTAMATE	ASSAY TYPE	GLUTAMATE	ASSAY TYPE
	PMOL / UG DRY WEIGHT					
HIPPOCAMPUS	16.5 ± 3.0 (3)	CYCLING	17.0 ± 2.6 (3)	CYCLING		
NUCLEUS ACCUMBENS	27.2 ± 11.3 (7)	ALKALI ENHANCED	26.2 ± 1.8 (3)	ALKALI ENHANCED	16.7 ± 6.4 (2)	ALKALI ENHANCED
CORTEX (ROSTRAL	20.5 ± 5.5 (6)	CYCLING	38.8 ± 18.2 (3)	CYCLING		
CORTEX (CAUDAL)	15.0 ± 2.5	CYCLING	24.8 ± 3.2 (3) *	CYCLING	22.3 ± 6.3 (4) *	CYCLING
GLOBUS PALLIDUS	39.8 ± 11.3 (5)	CYCLING			23.4 ± 9.4 (4)	CYCLING
NUCLEUS TRACTUS SOLITARIUS (ROSTRAL)	98.8 ± 49.4 (4)	CYCLING	36.1 ± 15.6 (5)	CYCLING		
NUCLEUS TRACTUS SOLITARIUS (CAUDAL)	77.2 ± 52.4 (3)	CYCLING	66.9 ± 9.7 (2)	CYCLING	44.5 ± 11.2 (5)	CYCLING
NUCLEUS AMBIGUOUS	24.6 ± 7.8 (3)	ALKALI ENHANCED	35.6 ± 10.9 (3)	ALKALI ENHANCED		
LOCUS COERULEUS	19.2 ± 7.6 (3)	ALKALI ENHANCED	18.4 ± 6.6 (5)	ALKALI ENHANCED		
CORPUS STRIATUM (ROSTRAL)	24.1 ± 7.6 (8)	CYCLING	18.7 ± 3.8 (3)	CYCLING	37.6 ± 5.8 (5) †	CYCLING
CAUDAL CAUDATE PUTAMEN	15.6 ± 6.4 (7)	CYCLING	22.8 ± 7.9 (7) †			
MEDIAL CAUDATE PUTAMEN	22.1 ± 6.3 (3)	CYCLING			19.5 ± 6.1 (4)	CYCLING
LATERAL CAUDATE PUTAMEN	23.6 ± 14.3 (4)	CYCLING			29.4 ± 9.1 (3)	CYCLING

VALUES ARE MEANS ± S.D OF THE NUMBER OF SECTIONS INDICATED IN PARENTHESES.

† $P < 0.10$ * $P < 0.05$

TABLE 3

ACETYLCHOLINESTERASE ACTIVITY IN SELECTED BRAIN REGIONS
OF SOMAN-TREATED AND NORMAL RATS

NUCLEUS	CONTROL	SOMAN 20 UG/KG	SOMAN 100 UG/KG
	NMOL · UG^{-1} · HR^{-1}		
NUCLEUS AMBIGUOUS	1.69 ± 0.13 (6)	2.12 ± 1.41 (10)	
NUCLEUS ACCUMBENS	2.41 ± 0.20 (4)	2.95 ± 0.13 (3) *	
CORTEX	1.2 ± 0.19 (5) 0.87 ± 0.39 (2)	0.85 ± 0.21 (4)	
LOCUS COERULEUS	6.15 ± 0.15 (3) 7.28 ± 0.39 (6)	4.91 ± 0.40 (5) * 4.91 ± 0.40 (5) **	
NUCLEUS TRACTUS SOLITARIUS	2.22 ± 0.43 (2)	2.28 ± 0.44 (7)	
ISLANDS OF CALLEJA	7.03 ± 1.33 (7)	4.10 ± 1.68 (8) **	4.68 ± 0.87 (6)
HYPOGLOSSAL NUCLEUS	2.30 ± 0.17 (5)	7.60 ± 0.34 (4) ***	2.64 ± 0.15 (7)
MEDIAL CAUDATE PUTAMEN	5.08 ± 0.62 (3)	8.42 ± 0.90 (5) *	NOT DETECTABLE.
LATERAL CAUDATE PUTAMEN	8.65 ± 2.07 (6)	7.64 ± 1.97 (6)	4.21 ± 0.84 (6) *
RETICULAR FORMATION	1.61 ± 0.28 (6)	NOT AVAILABLE	0.68 ± 0.14 (6) *

VALUES ARE MEANS ± S.E.M. OF THE NUMBER OF SAMPLES INDICATED IN PARENTHESES. ANIMALS RECEIVING 20 UG SOMAN/KG WERE KILLED 30 MIN AFTER ADMINISTRATION OF THE DRUG. ANIMALS RECEIVING 100 UG/KG WERE KILLED 10 MIN AFTER ADMINISTRATION OF THE DRUG.

* $P < 0.05$ *** $P < 0.001$
** $P < 0.01$

TABLE 4

PRESERVATION OF ACETYLCHOLINESTERASE ACTIVITY IN SPECIFIC NUCLEI OF THE BRAINS OF RATS TREATED WITH SOMAN

Nuclei	Control	20 ug/kg Soman	100 ug/kg Soman
		nmol · ug dry wt^{-1} · hr^{-1}	
		FOREBRAIN	
Lateral Caudate Putamen	13.07 ± 1.75 (3)	12.17 ± 0.85 (3)	4.89 ± 0.69 (5) ***
Medial Caudate Putamen	5.08 ± 0.62 (3)	8.42 ± 0.90 (6) *	Not detectable
Islands of Calleja	9.75 ± 1.02 (4)	14.76 ± 0.85 (7) **	4.68 ± 0.87 (6)
		BRAINSTEM	
Hypoglossal Nucleus	7.3 ± 0.17 (5)	7.60 ± 0.36 (4) ***	2.64 ± 0.15 (7)
Reticular Formation	1.61 ± 0.28 (6)	Not Available	0.68 ± 0.14 (6) *

Values are means ± S.E.M. of the number of samples indicated in parentheses.

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

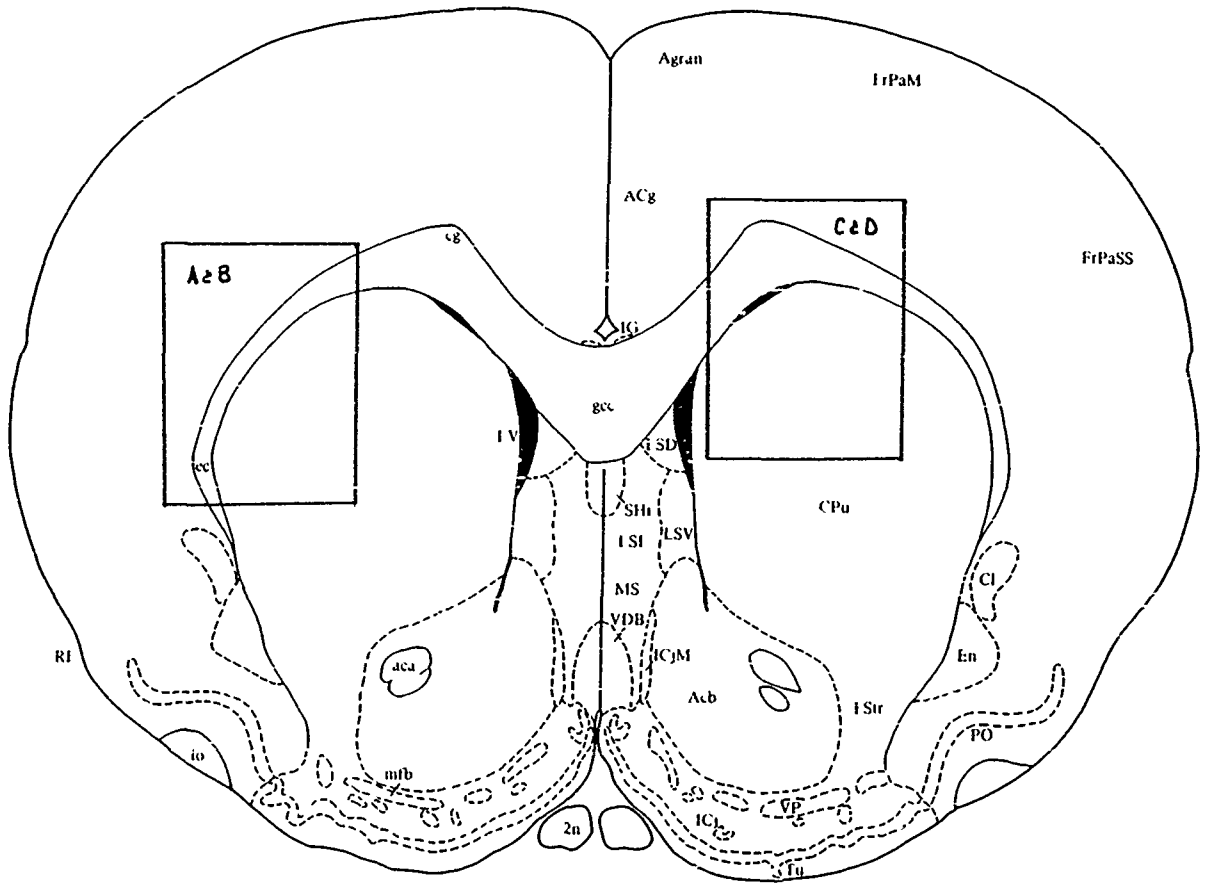


FIGURE 1

DIAGRAM OF A RAT FOREBRAIN SECTION, INDICATING IN BOXES THE AREAS PHOTOGRAPHED IN FIGURE 2. THE LATERAL CAUDATE PUTAMEN (CPU) AND THE MEDIAL ISLANDS OF CALLEJA (ICJM) RETAIN ACETYLCHOLINESTERASE STAINING AFTER TREATMENT WITH 100 UG/KG SOMAN (REPRODUCED FROM PAXINOS, G. AND WATSON, C., "THE RAT BRAIN IN STEREOTAXIC COORDINATES," ACADEMIC PRESS, NEW YORK, 1982).

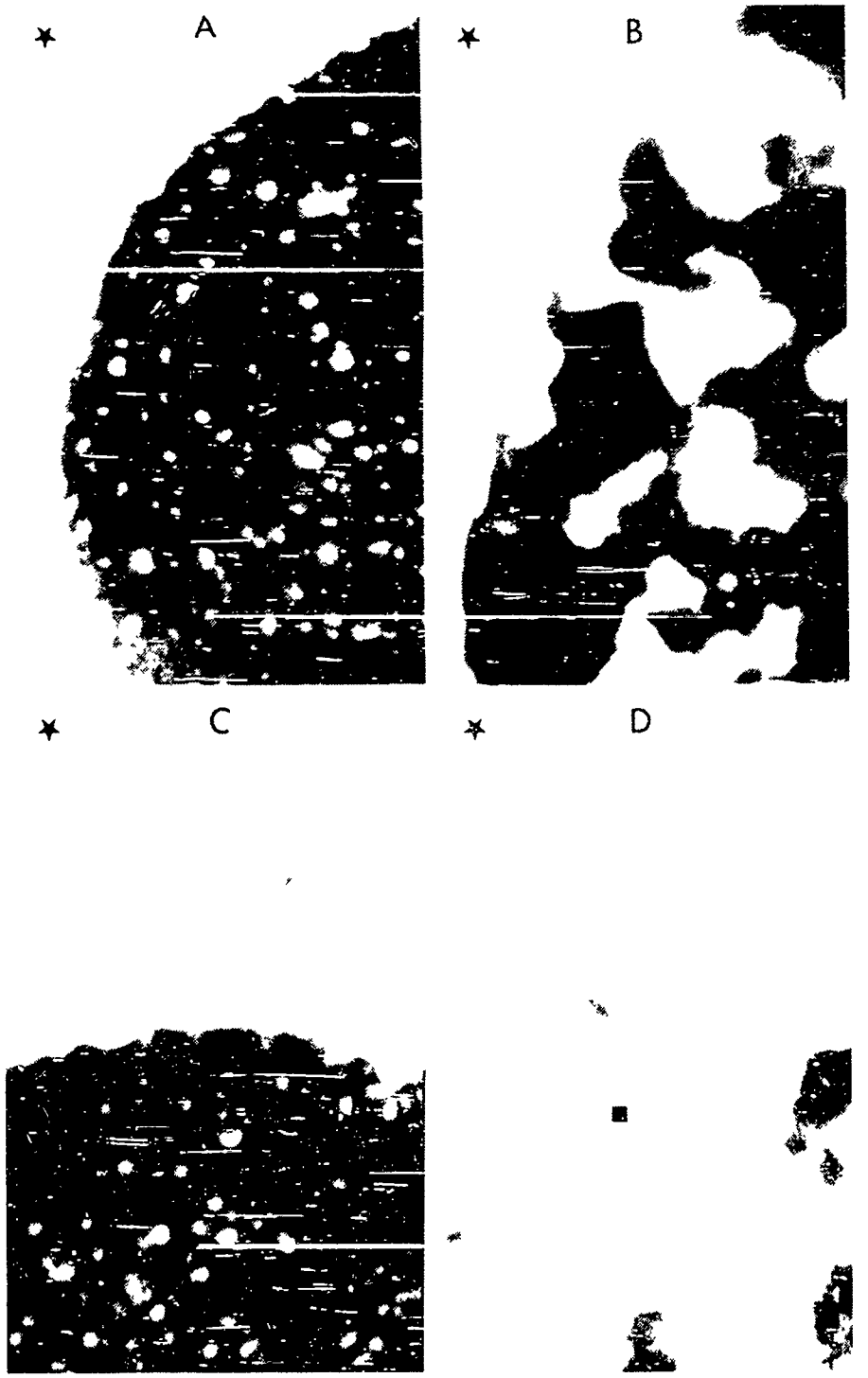


FIGURE 2

PHOTOMICROGRAPH OF AChE STAINED FOREBRAIN SECTIONS OF CONTROL ANIMALS (LEFT) AND OF ANIMALS TREATED WITH 100 UG/KG SOMAN (RIGHT):

A AND B. LATERAL CAUDATE PUTAMEN (DARK) AND CORTEX (*). UP - DORSAL; LEFT - LATERAL.

C AND D. MEDIAL CAUDATE PUTAMEN (DARK,■) AND CORTEX (*). UP - DORSAL; RIGHT - LATERAL (MAGNIFIED 30X).

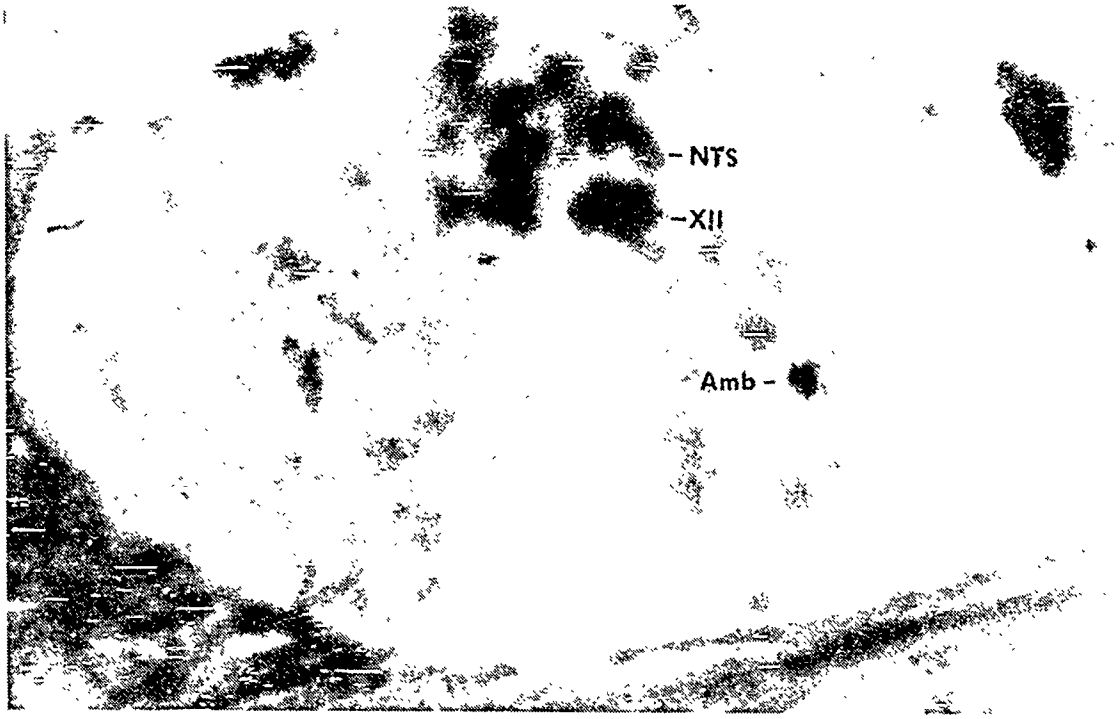


FIGURE 3

PHOTOMICROGRAPH OF AN AChE STAINED BRAINSTEM SECTION OF A RAT TREATED WITH 100 UG/KG SOMAN, EXHIBITING SOME OF THE AREAS RESISTANT TO THE DRUG: NUCLEUS AMBIGUOUS (AMB), HYPOGLOSSAL NUCLEUS (XII), AND NUCLEUS TRACTUS SOLITARIUS (NTS). (MAGNIFIED 30X).

CONCLUSIONS

1. SUBLETHAL DOSES OF THE DRUG ELEVATED THE GLUTAMATE CONCENTRATION BY 65-89% IN THE FRONTAL CORTEX. LETHAL DOSES OF THE DRUG ELEVATED THIS METABOLITE BY 48% (TABLE 2). ELEVATION OF GLUTAMATE, AN EXCITATORY AMINO ACID, COULD UNDERLIE THE CELLULAR DAMAGE OBSERVED IN THE CORTICES OF ANIMALS EXPOSED TO LETHAL DOSES OF SOMAN (McLOED ET AL., NEUROTOXICOL. 51: 53-58, 1984).

2. HISTOCHEMICALLY, NO DIFFERENCES IN AChE WERE DISCERNABLE BETWEEN THE CONTROL ANIMALS AND THOSE TREATED WITH A SUBLETHAL DOSE OF THE DRUG. IN CONTRAST, THE RADIOMETRIC AChE ASSAY INDICATED MARKED CHANGES: A DECLINE OF 53% IN THE LOCUS COERULEUS AND 48% IN THE ISLANDS OF CALLEJA, AND ELEVATIONS OF 66% AND 230% IN THE MEDIAL CAUDATE PUTAMEN AND HYPOGLOSSAL NUCLEUS, RESPECTIVELY (TABLE 3).

3. HIGH DOSES OF SOMAN INHIBITED COMPLETELY ACETYLCHOLINESTERASE ENZYMATIC ACTIVITY AND STAINING IN MOST AREAS OF THE BRAIN, BUT SOME AREAS WERE RESISTANT TO THE DEPLETION OF THE ENZYME BY THE TOXIN. THESE AREAS, WHICH STILL EXHIBITED INTENSE, BUT PATCHY CHOLINESTERASE STAINING WERE: THE LATERAL CAUDATE PUTAMEN, THE ISLANDS OF CALLEJA, THE NUCLEUS AMBIGUOUS, THE RETICULAR NUCLEUS, THE NUCLEUS OF THE XII NERVE, THE LOCUS COERULEUS AND THE RETICULAR FORMATION (TABLE 4).

4. THE ACTIONS OF SOMAN ON GLUTAMATE APPEAR HIGHLY SELECTIVE AND APPEAR RESTRICTED TO CEREBRAL CORTEX AND CAUDAL CAUDATE PUTAMEN. PRESERVATION OF AChE STAINING IN SOME BRAIN REGIONS OF ANIMALS TREATED WITH SOMAN MOST LIKELY REFLECTS DIFFERENCES IN DISTRIBUTION OF THE NEUROTOXIN.

EVALUATION OF THE EFFECTS OF ANTIDOTES TO SOMAN ON BRAIN CHOLINESTERASE ACTIVITY AND ACETYLCHOLINE LEVELS

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ABSTRACT

The toxicity of organophosphate cholinesterase (ChE) inhibitors is attributable to the inhibition of ChE activity and the subsequent elevation of acetylcholine (ACh) levels. Antidotes to these ChE inhibitors may alleviate the toxicity by reducing the degree of ChE inhibition and/or ACh elevation. We examined the effects of several antidotes, anticholinergics, anticonvulsants or oximes, to a potent organophosphate ChE inhibitor, soman, on brain ChE activity and ACh levels. Male rats were injected with atropine sulfate (16 mg/kg, i.m.), atropine methylnitrate (17 mg/kg, i.m.), aprophen (100 and 200 mg/kg, i.m.), diazepam (2.5 and 5.0 mg/kg, i.m.), 2-PAM (43 mg/kg, i.m.), or HI-6 (125 mg/kg, i.p.) in the presence or absence of soman (100 μg/kg, s.c.). Thirty minutes after injection, animals were killed by decapitation for ChE assay or by focused head microwave for ACh analysis, and brains were dissected into 6 parts: brainstem (B), cortex (C), hippocampus (H), midbrain (M), cerebellum (R), and striatum (S). Soman produced a 60% (S) to 80% (H, C) inhibition of ChE activity. The oximes, 2-PAM or HI-6, were unable to protect against soman-induced brain ChE inhibition, even though HI-6 treatment alone was beneficial in reversing the soman toxicity (Soc. Neurosci. Abstr. 10: 1184, 1984). Neither anticholinergics nor diazepam treatment affected ChE activity in any control brain area. Additionally, neither treatment reversed the ChE depression induced by soman. ACh levels were decreased by atropine sulfate and aprophen in all brain areas except B and R. In contrast, diazepam produced a dose-related increase of ACh in C, M and S. Therefore, the data suggest that atropine sulfate, aprophen, and diazepam changed brain ACh levels by a mechanism not related to ChE reactivation. Soman increased brain ACh levels by 6% (B) to 75% (C, H). Atropine sulfate did not reverse the soman-elevated ACh levels. Methyl atropine and oximes were not effective in modifying brain ACh levels of controls or in reversing the soman-induced ACh elevation. Although the relative ineffectiveness of these antidotes (except HI-6) in protecting rats against soman lethality may be related to their ineffectiveness in reversing the soman-induced brain ChE inhibition and ACh elevation, the observation of the effectiveness of HI-6 does not support such a relationship. Therefore, the measurement of brain regional ChE activity or ACh levels may not be useful in predicting the effectiveness of antidotes to soman poisoning. The study of other brain neurochemical functions of the cholinergic system, such as the turnover rate of ACh or the release of ACh from presynaptic terminals, may serve as a more specific indication of soman intoxication.

INTRODUCTION

1. The Toxicity of the Organophosphate Cholinesterase (ChE) inhibitor, Soman, is attributable to the inhibition of ChE activity and to subsequent elevation of acetylcholine (ACh) levels.

2. Soman intoxication is currently treated with a combination of anticholinergics, oximes and anticonvulsants to block cholinergic receptors, restore ChE activity and manage convulsions, respectively.

3. Effective protective compounds might also be expected to reduce elevated ACh concentrations.

PURPOSE

- To evaluate the effects of representative treatment compounds, in the presence/absence of soman intoxication, on ChE activity and ACh concentrations in various brain areas.

METHODS

1. ANIMALS

   Male, Sprague-Dawley × Wistar rats, weighing between 200 - 270 grams, were used.

2. DRUGS AND TREATMENT

   Groups of rats were treated with Atropine Sulfate (16 mg/kg, i.m.), Atropine methylnitrate (17 mg/kg, i.m.), Aprophen (100 and 200 mg/kg, i.m.), diazepam (2.5 and 5.0 mg/kg, i.m.), 2-PAM (43 mg/kg, i.m.), or HI-6 (125 mg/kg, i.p.) in the presence or absence of Soman (100 μg/kg, S.C.).

3. BRAIN TISSUE COLLECTION

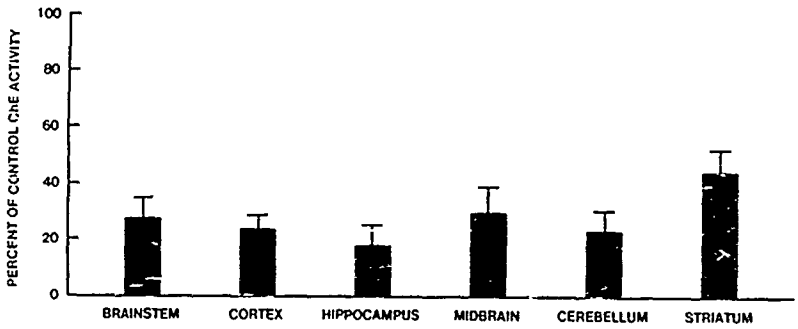
   Thirty minutes after injection, animals were killed by decapitation for ChE assay or by focused head microwave for ACh analysis. The brains were dissected into brainstem, cortex, hippocampus, midbrain, cerebellum, and striatum.

4. BRAIN ChE ASSAY

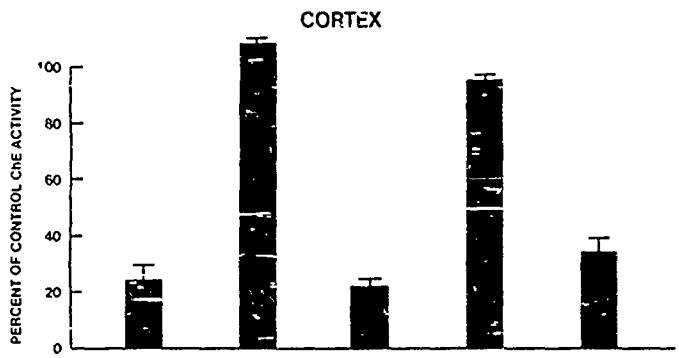
   ChE activity was assayed by the automated colorimetric method (Groff, et al., 1976).

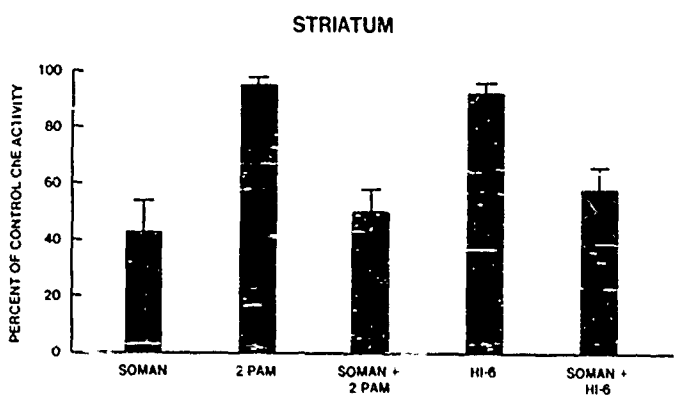
5. BRAIN ACh ANALYSIS

   Brain ACh levels were analyzed by the gas chromatograph/ mass spectrometric method (Shih, 1982).

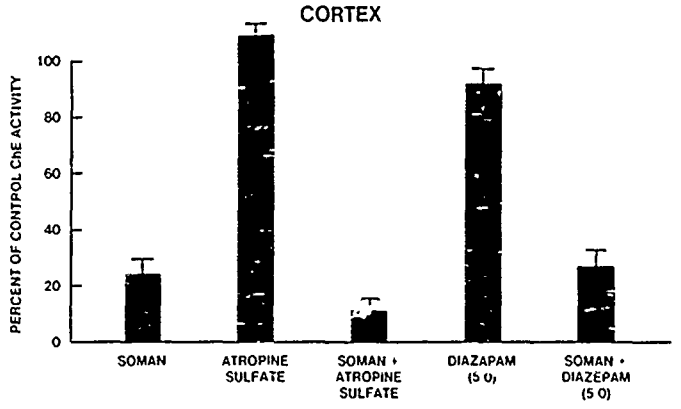


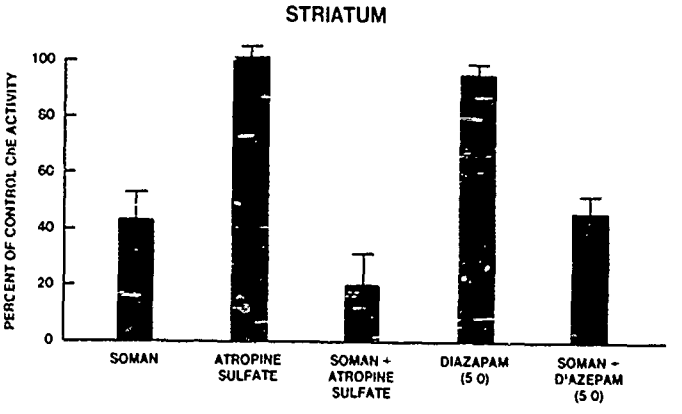
EFFECTS OF SOMAN (100 µg/kg, S.C.)
ON BRAIN REGIONAL ChE ACTIVITY





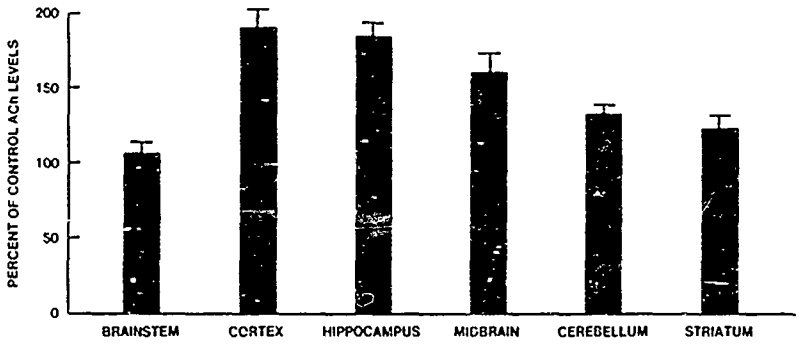
EFFECTS OF OXIMES ON BRAIN ChE ACTIVITY
IN THE PRESENCE OR ABSENCE OF SOMAN INTOXICATION



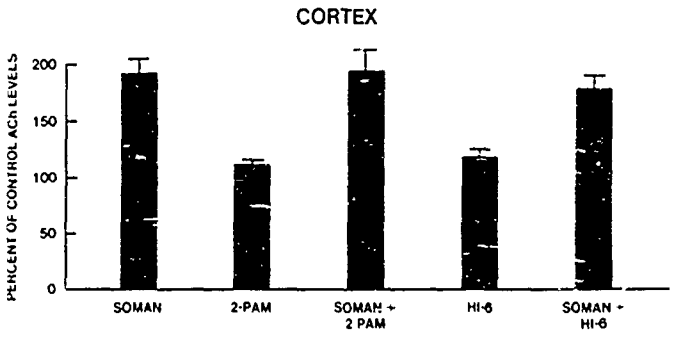


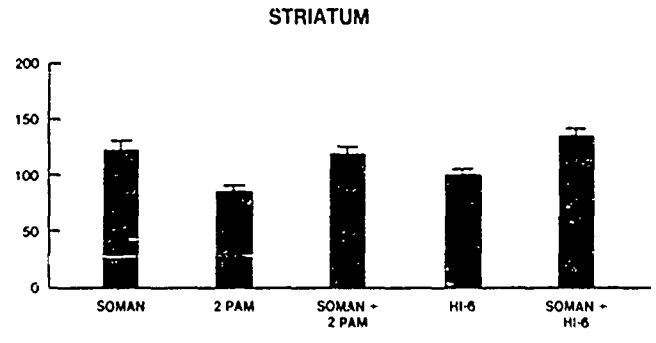
EFFECTS OF ATROPINE SULFATE OR DIAZAPAM ON BRAIN ChE ACTIVITY IN THE PRESENCE OR ABSENCE OF SOMAN INTOXICATION

ACh LEVELS

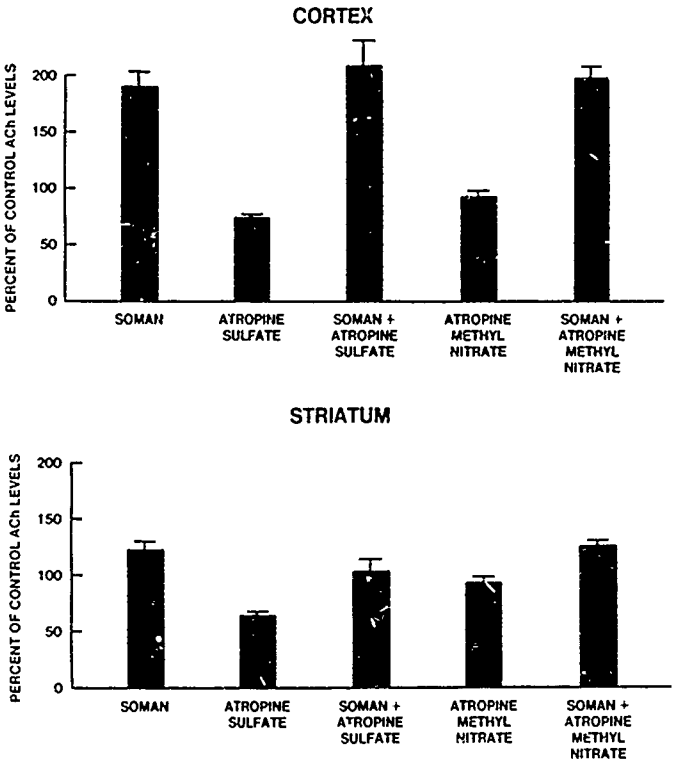


EFFECTS OF SOMAN (100 μg/kg, S.C.) ON BRAIN REGIONAL ACh LEVELS

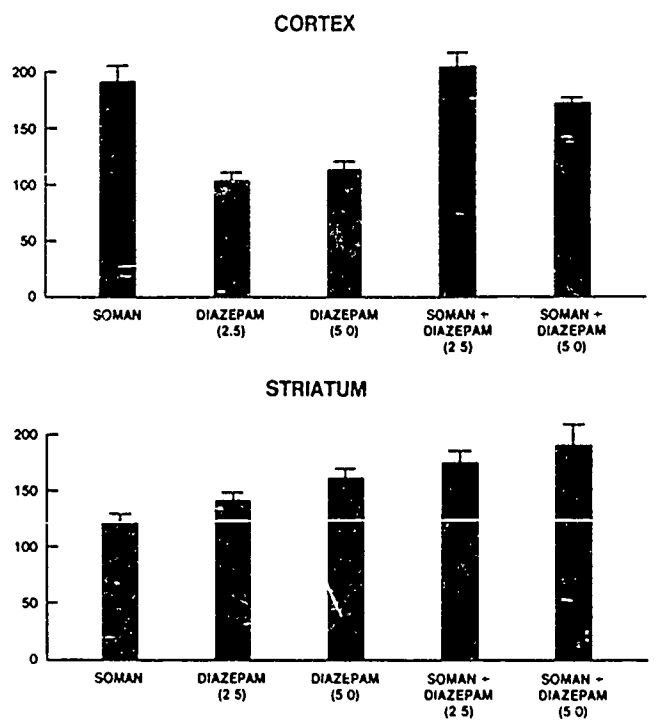




EFFECTS OF OXIMES ON BRAIN ACh LEVELS IN THE PRESENCE OR ABSENCE OF SOMAN INTOXICATION



EFFECTS OF ATROPINE SULFATE OR ATROPINE METHYLNITRATE ON BRAIN ACh LEVELS IN THE PRESENCE OR ABSENCE OF SOMAN INTOXICATION



EFFECTS OF DIAZEPAM ON BRAIN ACh LEVELS IN THE PRESENCE OR ABSENCE OF SOMAN INTOXICATION

CONCLUSIONS

1. Soman produced an expected depression of ChE activity and elevation of ACh concentrations in rat brain regions.

2. ChE activity was not affected by any treatment compound tested, either in the presence or in the absence of soman intoxication in any brain region examined.

3. ATROPINE SULFATE:
   - Reduces Ach concentrations in all brain areas examined in the absence of soman, but prevents elevated ACh concentrations in the presence of soman only in the striatum.

4. METHYL ATROPINE:
   - Does not affect ACh concentrations in the presence/absence of soman intoxication.

5. 2-PAM CHLORIDE AND HI-6:
   - Neither compound affects ACh concentrations in the presence/absence of soman intoxication despite the ability of HI-6 to protect against soman lethality.

6. DIAZEPAM:
   - Increases ACh concentrations both in the presence and absence of soman intoxication in the striatum.

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EFFECTS OF DIISOPROPYLFLUOROPHOSPHATE (DFP) ON RELEASE OF ENDOGENOUS NOREPINEPHRINE AND DOPAMINE FROM RAT BRAIN REGIONS

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INTRODUCTION

DFP is a neurotoxic organophosphate whose major mechanism of action is the irreversible inhibition of the enzyme acetylcholinesterase. The subsequent disruption of cholinergic central and peripheral neurotransmission is thought to be the primary cause of the physiological and behavioral perturbations observed following DFP exposure. While cholinergic synapses might be the major target of DFP, other neurotransmitter systems might also be involved in the acute and delayed effects of DFP. Recovery of acetylcholinesterase activity does not always parallel return to normal function.

The catecholamine neurotransmitters norepinephrine (NE) and dopamine (DA) have been shown to be involved in the central control of numerous critical physiological functions (e.g. sleep, eating, movement, blood pressure). Disruption of central NE and DA neurotransmission would seriously affect normal function. In this study, we have examined the effects of DFP on the release of endogenous NE and DA using a sensitive technique able to measure the small absolute amounts of NE and DA released from individual brain regions in vitro.

Both the spontaneous and potassium-stimulated release of NE and DA were measured in the presence of DFP at various concentrations. DFP markedly inhibited both the spontaneous and potassium-stimulated release of DA from the corpus striatum and the n. accumbens-olfactory tubercle. DFP also inhibited the spontaneous and potassium-stimulated release of NE from cortex, hypothalamus and n. accumbens-o. tubercle. Follow-up experiments demonstrated that the inhibition of endogenous catecholamine release by DFP could not be completely explained by indirect effects of increased acetylcholine.

METHODS

EXPERIMENT 1:

EFFECTS OF DFP ON RELEASE OF ENDOGENOUS NE AND DA

FROM INDIVIDUAL BRAIN REGIONS

1. RATS WERE SACRIFICED BY DECAPITATION AND THE FOLLOWING REGIONS WERE DISSECTED: CORTEX, STRIATUM, COMBINED N. ACCUMBENS-O. TUBERCULE AND HYPOTHALAMUS.
2. THE PIECES WERE INDIVIDUALLY WEIGHED, CHOPPED AND PLACED IN TUBES WITH 2ML OF COLD MODIFIED KREBS BUFFER.
3. THE TISSUE WAS CENTRIFUGED, WASHED, AND RECENTRIFUGED. THE PELLETS WERE RESUSPENDED IN 1ML BUFFER. KCL AND/OR DFP WAS ADDED TO SOME SUSPENSIONS. TUBES WERE INCUBATED AT 37° FOR 10 MIN.
4. RELEASE WAS TERMINATED BY CENTRIFUGATION AND THE SUPERNATANTS WERE ASSAYED FOR NE AND DA.

EXPERIMENT 2:

EFFECT OF ATROPINE OR MECAMYLAMINE

ON DFP INHIBITED RELEASE OF STRIATAL DA.

1. RATS WERE SACRIFICED BY DECAPITATION AND THE STRIATUM DISSECTED, WEIGHED AND CHOPPED.
2. THE POOLED TISSUE WAS WASHED TWICE THEN RESUSPENDED IN BUFFER.
3. ALIQUOTS OF THE TISSUE SUSPENSION WERE CENTRIFUGED, THE SUPERNATANTS THEN DISCARDED.
4. THE TISSUE PELLETS WERE PRE-INCUBATED WITH BUFFER ONLY OR ATROPINE (2A) OR MECAMYLAMINE (2B). DFP WAS ADDED 5 MIN LATER TO SELECTED TUBES.
5. KCL WAS ADDED TO ALL TUBES AFTER 5 ADDITIONAL MIN OF INCUBATION.
6. AFTER ANOTHER 10 MIN OF INCUBATION, RELEASE WAS TERMINATED BY CENTRIFUGATION AND THE SUPERNATANTS WERE ASSAYED FOR DA.

EXPERIMENT 3:

EFFECT OF DIFFERENT CONCENTRATIONS OF DFP, PHYSOSTIGMINE,

ATROPINE, MECAMYLAMINE, OXOTREMORINE OR NICOTINE

ON RELEASE OF STRIATAL DA.

1. STRIATAL POOLED TISSUE WAS PREPARED AS IN EXP 2 ABOVE.
2. INCUBATIONS WERE PERFORMED WITH THE FOLLOWING DRUGS: DFP, PHYSOSTIGMINE SULFATE, ATROPINE SULFATE, MECAMYLAMINE HYDROCHLORIDE, OXOTREMORINE SESQUIFUMARATE, NICOTINE BITARTRATE, IN CONCENTRATIONS OF 10^{-3}, 10^{-4} OR 10^{-5}M.
3. AFTER 5 MIN KCL WAS ADDED TO SOME TUBES.
4. TUBES WERE CENTRIFUGED AFTER 10 ADDITIONAL MIN OF INCUBATION AND THE SUPERNATANTS ASSAYED FOR DA.

FIG 1 : EFFECT OF DFP ON NE RELEASE FROM
RAT BRAIN REGIONS IN VITRO
*differs from control, $p<0.05$

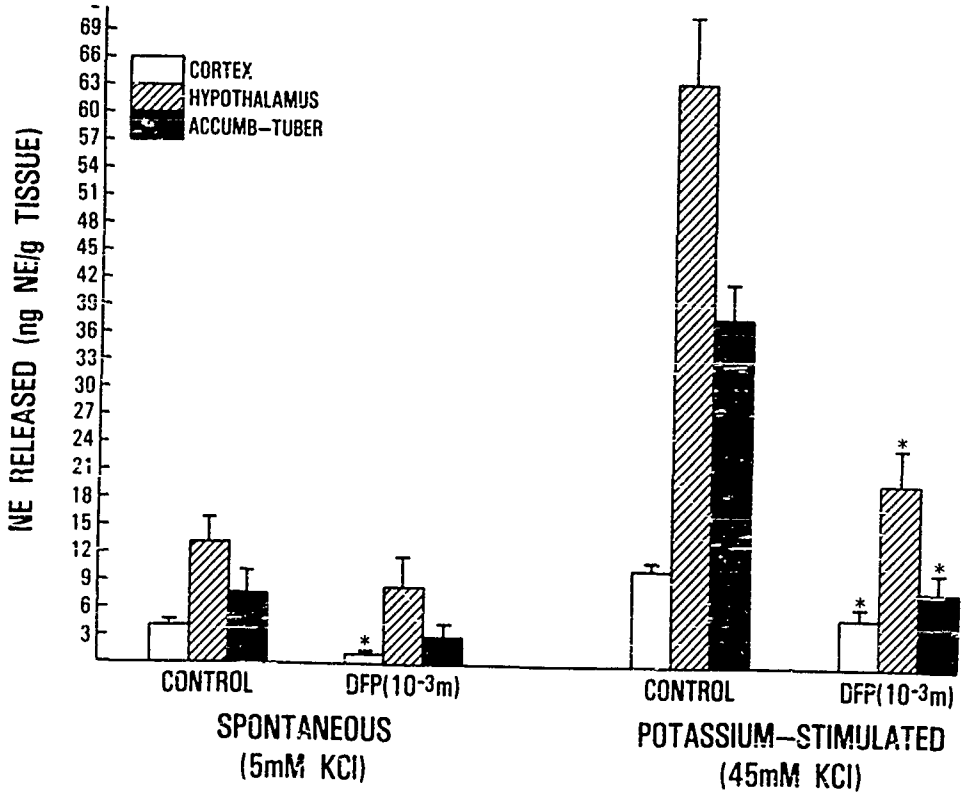

FIG 2 : EFFECT OF DFP ON DA RELEASE FROM
RAT BRAIN REGIONS IN VITRO
*differs from control, $p<0.05$

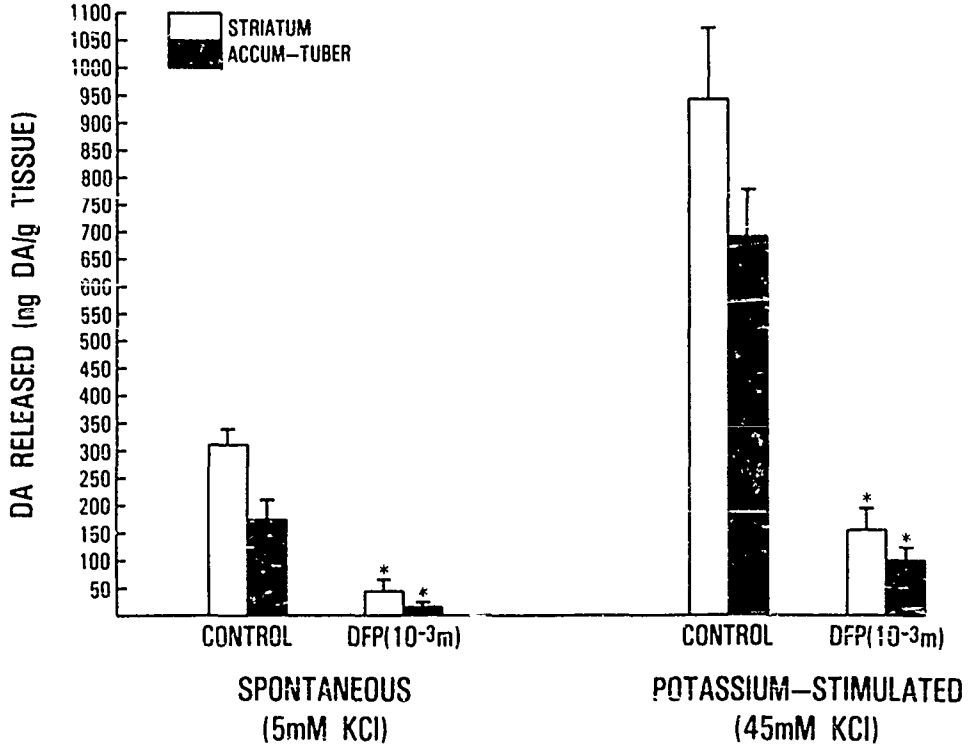

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FIG 3 : EFFECT OF ATROPINE OR MECAMYLAMINE ON DFP INHIBITION OF STRIATAL DA RELEASE
*differs from appropriate control, p<0.05

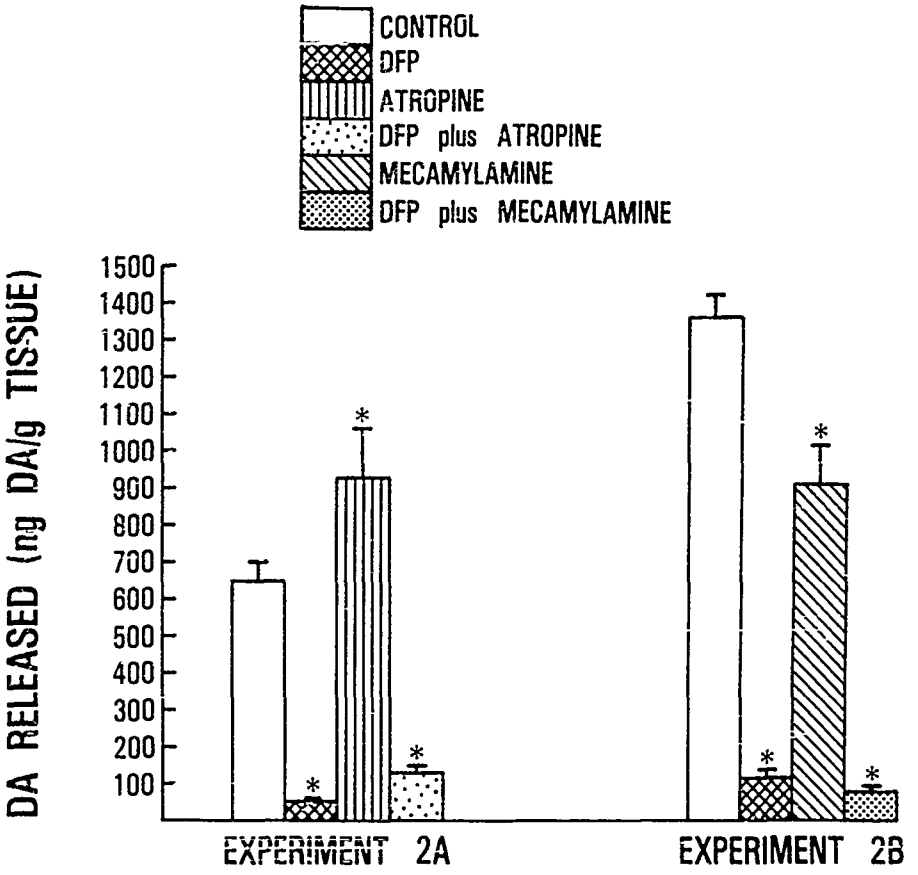

FIG 4 : EFFECTS OF DFP AND PHYSOSTIGMINE ON SPONTANEOUS RELEASE OF DA FROM STRIATUM IN VITRO
*differs from appropriate control, p<0.05

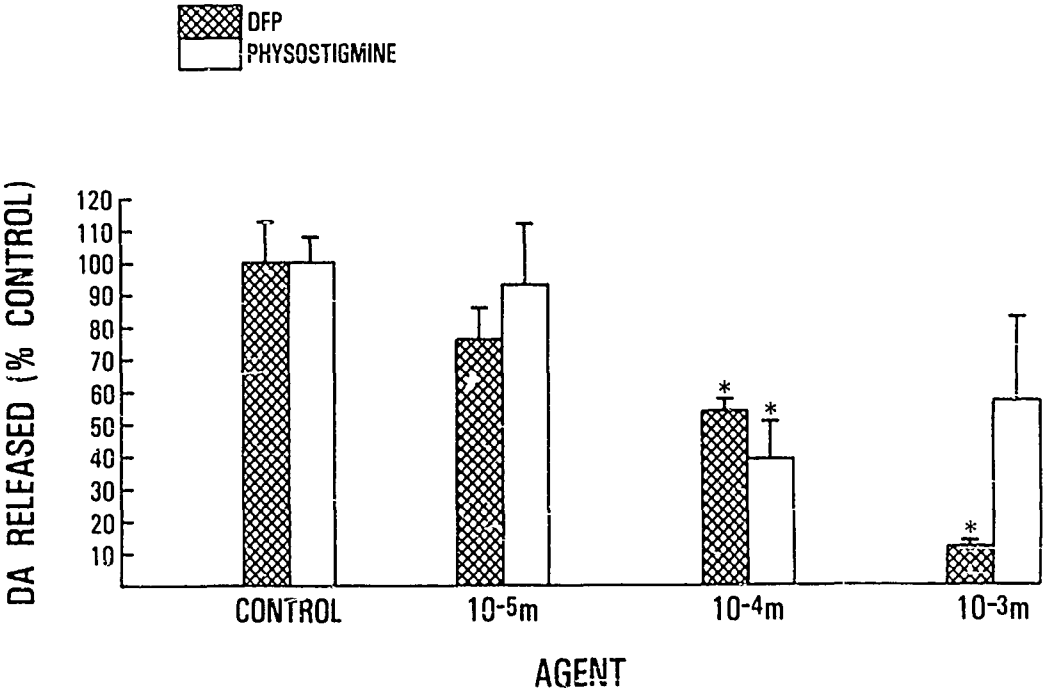

FIG 5: EFFECTS OF CHOLINERGIC AGENTS ON SPONTANEOUS RELEASE OF DA FROM STRIATUM IN VITRO

Agent	DA released (% control) Control[1]	10^{-5}M	10^{-4}M	10^{-3}M
Atropine	100 ± 14	87 ± 12	49 ± 13*	136 ± 26
Mecamylamine	100 ± 07	106 ± 11	104 ± 09	157 ± 16*
Oxotremorine	100 ± 19	132 ± 18	98 ± 31	132 ± 16
Nicotine	100 ± 28	91 ± 14	175 ± 39	289 ± 34*

Values represent the mean ± SEM of 6 samples. KCl concentration (5mM) for all tubes. [1]Separate control tubes were incubated for each drug since each incubation set of 24 tubes was derived from a separate tissue pool. Average DA released in controls was 380 ng DA released per gram striatal wet weight. The 24 samples representing control plus 3 concentrations of drug were incubated and assayed together. Comparisons are made within incubations.

*Differs from appropriate control, $P < 0.05$, Student's t test, 2-tailed.

FIG 6: EFFECTS OF DFP AND PHYSOSTIGMINE ON POTASSIUM-STIMULATED RELEASE OF DA FROM STRIATUM IN VITRO
***differs from appropriate control, p<0.05**

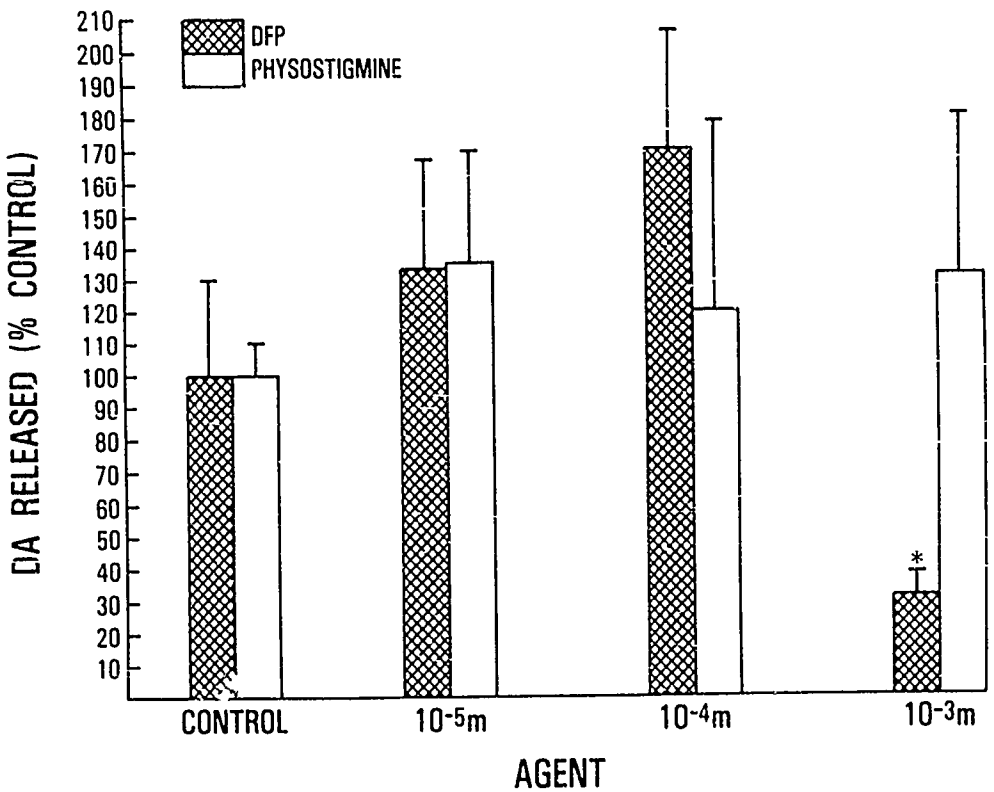

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FIG 7: EFFECTS OF CHOLINERGIC AGENTS ON POTASSIUM-STIMULATED RELEASE OF DA FROM STRIATUM IN VITRO

	DA released (% Control)			
Agent	Control[1]	10^{-5}M	10^{-4}M	10^{-3}M
Atropine	100 ± 06	111 ± 07	116 ± 04*	78 ± 09*
Mecamylamine	100 ± 14	95 ± 26	109 ± 14	118 ± 26
Oxotremorine	100 ± 21	68 ± 13	73 ± 15	56 ± 08*
Nicotine	100 ± 11	120 ± 16	109 ± 12	127 ± 08*

Values represent the mean ± SEM of 6 samples. KCl concentration (45mM).

[1]Separate control tubes were incubated for each drug since each incubation set of 24 tubes was derived from a separate tissue pool. Average control DA release was 1433 ng DA released per gram striatal wet weight. The 24 samples representing control plus 3 concentrations of drug were incubated and assayed together. Comparisons are made within incubations. * Differs from appropriate control, $P<0.05$, Student's t test, 2-tailed.

CONCLUSIONS

1. USING A SENSITIVE RADIOISOTOPE-ENZYMATIC TECHNIQUE, WE WERE ABLE TO MEASURE THE SMALL ABSOLUTE AMOUNTS OF CATECHOLAMINES RELEASED FROM INDIVIDUAL BRAIN REGIONS.

2. INCREASED POTASSIUM CONCENTRATION INCREASED RELEASE OF NE AND DA AS EXPECTED. KCL CAUSES DEPOLARIZATION OF NEURONAL TISSUE AND STIMULATES NEUROTRANSMITTER RELEASE.

3. DFP MARKEDLY INHIBITED THE RELEASE OF ENDOGENOUS NE AND DA FROM THE REGIONS EXAMINED. BOTH SPONTANEOUS AND POTASSIUM-STIMULATED RELEASE WERE REDUCED BY DFP BUT THE EFFECT ON POTASSIUM-STIMULATED RELEASE WAS SEEN AT LOWER DFP CONCENTRATIONS AND WAS DOSE RELATED.

4. NICOTINE INCREASED DA RELEASE. THEREFORE DFP INHIBITION OF DA RELEASE IS NOT MEDIATED VIA STIMULATION OF NICOTINIC RECEPTORS BY EXCESS ACH.

5. AT 10^{-5}M AND 10^{-4}M CONCENTRATIONS, THE INHIBITORY EFFECTS OF PHYSOSTIGMINE AND OXOTREMORINE ON POTASSIUM-STIMULATED DA RELEASE WERE SIMILAR IN MAGNITUDE TO THOSE OF DFP.

6. HOWEVER, THE MARKED INHIBITORY EFFECTS OF 10^{-3}M DFP ON BOTH SPONTANEOUS AND POTASSIUM-STIMULATED DA RELEASE WERE NOT SEEN FOLLOWING EITHER PHYSOSTIGMINE OR OXOTRMORINE. THE EFFECTS OF 10^{-3}M DFP WERE ONLY SLIGHTLY BLOCKED BY ATROPINE.

7. TAKEN AS A WHOLE, THESE DATA SUGGEST THAT AT MODERATE CONCENTRATIONS, DFP EFFECTS ON DA RELEASE MAY BE DUE TO EFFECTS OF INCREASED ACH AT MUSCARINIC RECEPTORS ON DA NEURONS. HOWEVER AT HIGH DFP (10^{-3}M) CONCENTRATIONS, DFP APPEARS TO INHIBIT RELEASE TO A GREATER EXTENT THAN CAN BE EXPLAINED BY THE PRESENCE OF EXCESS ACH.

COMPARISON OF THE TOXIC MANIFESTATIONS OF SOMAN (GD) AND ECHOTHIOPHATE (EC) IN MICE, AND THE PROTECTION AFFORDED BY CLONIDINE

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Introduction

GD and EC are potent irreversible cholinesterase inhibitors whose toxic effects involve several cholinergic systems. EC exerts most of its actions through peripheral systems since it does not readily cross the blood brain barrier. GD, on the other hand, affects both central and peripheral cholinergic systems, although it is not clear whether the primary site for acute lethality resides in the central or peripheral nervous system. The purpose of this study was to compare symptoms of GD and EC poisoning and to examine the potential protective actions of clonidine, a central alpha$_2$-adrenergic agonist, which has been shown to offer protection against physostigmine-, but not neostigmine-, induced toxicity (J. Pharmacol. Exp. Ther. 222:595, 1982).

Results and Discussions

1. The LD curves for GD and EC covered very narrow dose ranges (0.1-0.2 mg/kg; Tables 1 and 2). The most important difference in the toxicological profile of symptoms for the two compounds was the presence of whole body tremor and absence of muscle fasiculations in GD-treated animals and the absence of tremor and presence of muscle fasiculations in EC-treated animals. Thus, central and peripheral symptoms of toxicity predominated in GD- and EC-treated mice, respectively.

2. Clonidine is a centrally-acting alpha$_2$-adrenergic agonist which provides protection primarily against the central toxic manifestations of cholinesterase inhibitors. Pretreatment with clonidine reduced lethality, OTT, ST and SAL in GD-treated mice (Table 3). On the other hand, clonidine did not reduce lethality, MF, ST, or SAL in EC-treated mice (Table 4). There was, however, one unexpected finding in mice pretreated with clonidine: LRR in EC-treated mice was increased by clonidine. While this increase was not as great as in GD-treated animals, it was significant.

3. In order to account for this weak protective action of clonidine in EC-treated animals, we investigated the possibility that clonidine interacts directly with acetylcholinesterase to protect it from irreversible phosphorylation by the inhibitor. We found that, in clonidine pretreated mice, GD was, in fact, significantly less effective in reducing brain cholinesterase activity (Table 5).

4. The mechanism by which clonidine affords this protection of the enzyme in vivo is suggested by the experiments illustrated in Figure 1. Mouse brain cholinesterase activity (assessed in vitro) was inhibited by clonidine in a dose-dependent manner. Inhibition was apparent at 100 uM clonidine. Brain homogenates in which enzyme activity was reduced with GD prior to the start of the assay exhibited greater activity if the homogenate was incubated with 100 uM clonidine prior to the addition of GD.

5. These results indicate that 1) subcutaneous injection of GD in the mouse produces lethality primarily by central actions, 2) the protective effects of clonidine against peripherally-acting acetylcholinesterase inhibitors are minimal, and 3) the weak protection against the peripherally-acting inhibitors may be related to a weak inhibition of acetylcholinesterase by clonidine. Thus, clonidine may protect against cholinesterase inhibitor toxicity by two distinct mechanisms.

Table 1

ECHOTHIOPHATE TOXICITY IN THE MOUSE

DOSE mg/kg	n	LETHALITY %	LRR min	MF %	ST %	SAL %
0.100	8	0	-	0	0	0
0.125	16	25	18.4±3.0	43	6	42
0.150	22	45	15.0±1.5	100	14	100
0.175	16	94	12.9±1.0	100	6	100
0.200	14	100	10.1±0.4	100	0	100

LD50 = 0.141 mg/kg; LRR = Time to Loss of Righting Reflex; MF = Occurrence of Muscle Fasiculations; ST = Occurrence of Straub Tail; SAL = Occurrence of Salivation.

Table 2

SOMAN TOXICITY IN THE MOUSE

DOSE mg/kg	n	LETHALITY %	OTT min	LRR min	ST %	SAL %
0.100	10	0	5.8±0.71	-	40	0
0.125	7	0	3.5±0.31	-	71	29
0.150	10	40	3.7±0.47	100±20	100	80
0.175	12	83	3.5±0.36	9±1	92	83
0.200	12	100	3.4±0.31	10±1	100	100

LD50 = 0.156 mg/ml; OTT = Time to Onset of Tremor; LRR = Time to Loss of Righting Reflex; ST = Occurrence of Straub Tail; SAL = Occurrence of salivation.

Table 3

INFLUENCE OF CLONIDINE ON SOMAN TOXICITY IN THE MOUSE

CLONDINE mg/kg	n	LETHALITY %	OTT min	LRR min	ST %	SAL %
0	16	88	3.2±0.3	8.0±0.5	81	75
0.3	8	50	9.9±2.4*	11.0±7.5	50	50
1.0	7	14	17.7±2.1**	N	29	14

Clonidine was administered 20 min prior to 0.175 mg/kg Soman.

*p<0.05; **p<0.01 compared to non-pretreatment controls

N, insufficient data for statistical analysis.

Table 4

INFLUENCE OF CLONIDINE ON ECHOTHIOPHATE TOXICITY IN THE MOUSE

ECHOTHIO-PHATE mg/kg	CLONIDINE mg/kg	n	LETHALITY %	LRR min	MF %	ST %	SAL %
0.155	0	22	45	15.0±1.5	100	14	100
0.155	0.3	7	88	39.3±7.0*	100	0	100
0.155	1.0	7	71	51.9±5.9	100	14	100
0.175	0	16	94	12.9±1.0	100	6	100
0.175	0.3	8	100	18.4±1.6*	100	25	100
0.175	1.0	8	100	19.1±1.1*	100	38	100

Clonidine was administered 20 min prior to echothiophate.

*p<0.05 compared to nonpretreated controls.

Fig. 1

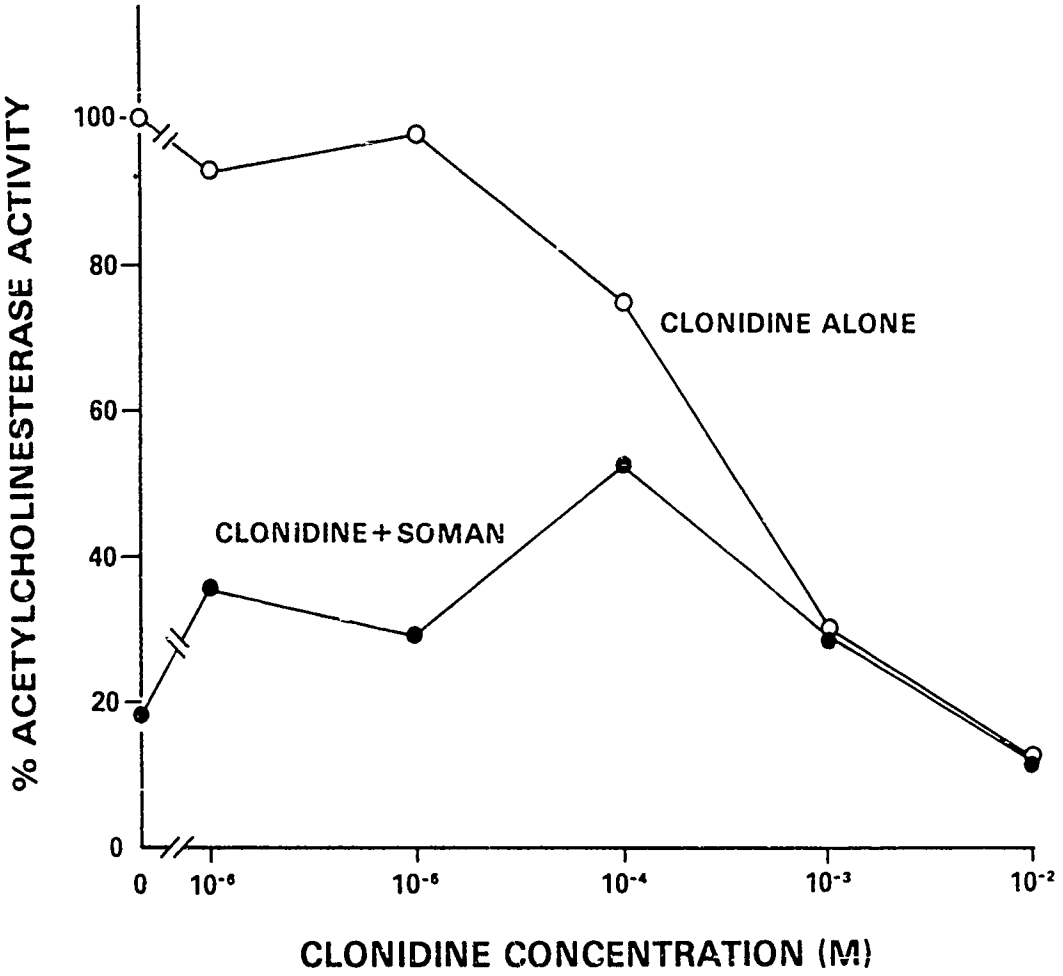

FIGURE 1. Influence of clonidine and Soman on mouse brain acetylcholinesterase activity in vitro. The indicated concentration of clonidine was incubated with an aliquot of whole brain homogenate for 15 min. The brain tissue was then incubated for a further 5 min in the absence (O) or presence (●) of Soman. Sufficient Soman was used to reduce enzyme activity to 18% of control levels. Each point represents the mean fdrom at least 6 determinations which varied by less than 15%.